THE PRODUCTION OF AUTOLOGOUS HUMAN MONOCLONAL ANTIBODIES WITH REACTIVITY AGAINST HUMAN BREAST CARCINOMA CELLS

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INDEX OF CONTENTS.

INDEX OF TABLES

INDEX OF PLATES.

INDEX OF FIGURES.

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SUMMARY

This thesis describes work which I performed as a research fellow in the Dept of Clinical Surgery, Royal Infirmary of Edinburgh, under the supervision of Mr O.Eremin esq., Senior Lecturer, studying the production of autologous human monoclonal antibodies with reactivity against human breast carcinoma cells. A review of the relevant literature concerning the immunology of breast cancer and the production and uses of monoclonal antibodies was performed.

In the experimental work, axillary node lymphocytes from patients undergoing surgery for carcinoma of the breast were fused with the human myeloma cell line LICR/LON/HMy2. Resulting Hybridomas were characterised by performing chromosome counts and investigating cell surface markers. Supernatants of these hybridomas were investigated for the presence of immunoglobuliun and the reactivity of that immunoglobulin with breast carcinoma cells investigated. Efforts were made to improve the rate of hybrid production by introducing four variables into the fusion experiment, viz. the prestimulation of lymphocytes with pokeweed mitogen, the addition of feeder cells, the use of azaserine in the selective medium, and the alteration of cell numbers. Of these only the addition of azaserine was seen to improve the rate of hybrid production. The results of this work are discussed in relationship to the published literature.

ABBREVIATIONS USED.

AEC	3-Amino 9-diethyl Carbizole.
DMSO	Dimetyhyl Sulphoxide.
EBV	Epstein Barr Virus
FCS	Foetal Calf Serum.
HAT	Hypoxanthine,Aminopterin and Thymidine.
HAzT	Hypoxanthine,Azaserine and Thymidine.
Ig	Immunoglobulin.
McAb	Monoclonal Antibody.
NSS	Normal Swine Serum.
PAP	Peroxidase anti-peroxidase Complex.
PBS	Phosphate Buffered Saline.
PEG	Polyethylene Glycol.
PWM	Pokeweed Mitogen.
SAR	Swine anti-rabbit immunoglobulin Antibody.
SIg	Surface immunoglobulin.
SRBC	Sheep Red Blood Cell.
TBS	Tris Buffered Saline
TCM	Tissue Culture Medium

SECTION 1.

AIMS OF THE THESIS.

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The aim of this study was to investigate the production of autologous human monoclonal antibodies (McAbs) with reactivity against breast carcinoma cells by the cellular fusion of axillary node lymphocytes from patients undergoing surgery for breast cancer with the human myeloma cell line LICR/LON/HMY2 (hereafter designated Hmy2).

Firstly, this study aimed to investigate ways in which the efficiency of hybridoma production might be improved, by investigating the beneficial effects of four variations of the fusion experiment. These variations are 1) the prestimulation of lymphocytes with pokeweed mitogen (PWM),2) the addition of feeder cells to the fused cell culture 3) the use of an alternative selective medium and 4) variation of the cell numbers used in the fusion experiment.

Secondly, this study aimed to investigate the means by which hybridomas and their secreted antibodies might be most appropriately characterised.

SECTION 2.

INTRODUCTION AND REVIEW OF THE LITERATURE.

2.1. Breast Cancer. A Brief Review of the Clinical Problem.

Carcinoma of the breast is a major cause of morbidity and mortality in females living in industrialised affluent western societies . In Scotland, 1175 women died of breast cancer in 1981 , this accounting for approximately 20% of all female cancer deaths (Anon, 1981). During the years 1975-1979 over 2000 new cases of breast cancer were registered annually, accounting for about 20% of all female cancer registrations (Anon,1981). It has been estimated that 1 in 14 women in the western world will suffer from breast cancer at some stage in their lives(Baum 1981). The methods used to treat this disease have increased as understanding of the natural history of breast cancer has evolved during the course of this century. These methods are briefly summarised as follows.

2.1.1.Surgery.

A variety of operations have been employed as primary treatment of breast cancer. Surgery has aimed to remove the tumour together with a variable amount of surrounding tissue (breast, muscle), as well as a variable number of the axillary nodes.

Halsted (1897) described in detail the operation of radical mastectomy which still bears his name. This operation consisted of the removal of the breast ,the underlying pectoral muscles and the axillary contents. Halsted introduced this radical operation in an attempt to reduce the high rate of local

recurrence associated with more conservative surgery at that time. This operation became standard treatment for carcinoma of the breast for many years.

This form of radical surgery was based on the premise that breast cancer spread initially from the primary tumour to the axillary nodes and finally to distant sites as metastases. As is discussed later in this chapter this is now thought not to be the case, but rather breast cancer is thought to disseminate early in the course of the disease . Hence the surgical treatment of breast cancer has become less radical and the operations of modified radical and simple mastectomy and , more recently, lumpectomy , have superceded radical mastectomy as the operations of choice for primary breast cancer.

2.1.2.Radiotherapy

Radiotherapy was the next mode of treatment introduced into breast cancer therapy and was originally used as an adjuvant to radical mastectomy. McWhirter et al. (1948) proposed that radical radiotherapy was an equally suitable method of treating axillary lymph nodes as surgical removal and,therefore,suggested simple mastectomy followed by axillary radiotherpy as an alternative to radical mastectomy. This regime was adopted by many surgeons in this country but not in the U.S.A. These techniques were employed in the belief that carcinoma of the breast spread radially outwards and that it would be possible,by aggressive local treatment, to remove all tumour cells during the early stages of the disease before distant metastases occurred.

This theory was challenged when long term follow up studies of patients were published. Notable amongst these was the work of Brinkley and Haybittle (Brinkley et al.,1975) who showed that women who had undergone radical mastectomy for clinically localised breast cancer had an increased incidence of death from metastatic disease up to 30 years post surgery. This and other studies laid the foundation of our current understanding of the behaviour of breast cancer as a disease which disseminates early in the course of the disease although actual distant recurrences may not appear for many years subsequently (Forrest,1981). Thus it became clear that improved survival would not come about by employing increasingly aggressive local therapy but that some form of systemic therapy may also be required to treat tumour cells which had disseminated early in the course of the disease.

2.1.3. Endocrine therapy.

Beatson (1896) first described oophorectomy in the treatment of patients with advanced or recurring breast cancer with apparent beneficial results. A relationship between carcinoma of the breast and the ovaries therefore was established. Recently,Jensen et al. (1971) demonstrated that some breast cancers contained cytoplasmic (and probably nuclear) receptors for oestrogen which were comparable to receptors found in other oestrogen sensitive tissues.This receptor site is a cytoplasmic protein which

binds oestrogen and translocates the hormone into the nucleus where it promotes cell protein synthesis and cell division.These discoveries have led to a wide variety of endocrine manipulations being employed in breast cancer therapy, including oophorectomy (Henley 1947), adrenalectomy (Higgins et al.,1953), hypophysectomy (Forrest et al.,1955), and the administration of drugs with hormone related activity e.g. the anti-oestrogen, tamoxifen.

2.1.4.Chemotherapy

Chemotherapy using cytotoxic agents has been widely used in the treatment of advanced breast cancer, usually employing a combination of such agents (Bisel 1980). More recently peri-operative chemotherapy has been advocated as adjuvant treatment in patients with operable breast cancer in an attempt to decrease the incidence of tumour recurrence.(Rossi et al 1981).

In summary, the treatment of breast cancer has become more complex. Factors such as the histological type and grade of the tumour, the degree of nodal invasion by the tumour,the measurement of oestrogen receptor status and the careful preoperative search for distant metastases may all be taken into account when planning treatment programmes for individual patients. The conducting of properly controlled clinical trials allows the merits of each form of treatment to be accurately assessed.

2.1.5.Survival Rates.

What effect have these alterations to the treatment of primary breast carcinoma made to the survival of patients suffering from this disease? When discussing survival data it is important

to bear in mind the concept that breast cancer is a disease which disseminates early but may recur late. Thus Brinkley and Haybittle (Brinkley et al 1975) recorded an 82.8% five year survival (age corrected) in a group of 704 patients with operable stage I disease, but this figure dropped to 46% at 25years, many of these patients dying of the disease.

Comparable results were obtained by Langlands (Langlands et al 1979) from a bigger series of 3878 patients. The benefits of adjuvant chemotherapy have yet to be assessed on a long term basis.

2.1.6.Screening.

One method of improving this gloomy outlook has been to attempt to detect the disease at an earlier stage using large scale screening programmes of the population at risk. Such programmes involve clinical and mammographic examinations and are currently under evaluation. Shapiro (1977) concluded from the initial results of the Health Insurance Plan breast screening programme in New York that screening might improve the prognosis of breast cancer patients under 50 years of age although the results in the older population were as yet uncertain. Comparable screening programmes are taking place at selected centres in the U.K. including Edinburgh , but the effects of breast screening on the overall mortality of breast cancer remains unclear.

Whatever the results of these screening programmes the fact remains that a large number of women presenting with breast cancer are still going to die of the disease. There is therefore a strong case in favour of exploring new avenues of treatment of this disease.

2.2.EVIDENCE OF AN IMMUNE RESPONSE TO BREAST CANCER.

There is a considerable volume of clinico-pathological and experimental evidence to suggest that human breast carcinoma provokes an immune response in the host individual. This response is thought to involve both cellular and humoral immune mechanisms. This chapter will outline some of the studies characterising this response as it relates to the work of this thesis.

2.2.1 Cell-Mediated Immune Response

Evidence of a cell-mediated immune response to breast carcinoma cells comes from a variety of in-vitro and in-vivo experiments, including skin hypersensitivity to tumour cell extracts,the use of a "skin window" to directly examine and grade the cellular response and the inhibition of leucocyte migration/adhesion by tumour cell extracts. For ethical reasons skin hypersensitivity tests and skin window tests cannot be used on healthy subjects as controls. This does to some extent make the results of these tests more difficult to evaluate.

Skin hypersensitivity tests involve injecting preparations of tumour cells intradermally and then measuring the degree of reaction produced by this injection. This gives a measure of the cell mediated response to the injected material. Hollinshead et al. (1974) used a skin hypersensitvity test to determine the cell mediated immune response to extracts of breast cancer cells. Soluble extracts from both breast carcinomas and normal

breast tissue were separated firstly on a Sephadex column and secondly by polyacrylamide gel electrophoresis. These extracts were then injected into both breast cancer patients and control patients suffering from non-breast,non-gynaecological cancers. It was found that one of the fractions obtained on the Sephadex column produced a positive reaction in breast cancer patients whether derived from breast carcinoma or normal breast.Further separation of this fraction however by electrophoresis isolated a protein fraction from breast carcinomas which produced a positive skin reaction whereas the equivalent fraction from normal breast did not. In addition the tumour fraction produced a positive reaction in the control patients whereas the fraction derived from normal breast did not. This study showed that breast cancer cell extracts could produce positive skin reactions in patients with breast and other cancers. This study has the disadvantage that only a small number of patients were included in the study.

Black et al.(1973) used a skin window technique to assess the reaction to tissue sections of autologous tumour. An abrasion was made on the patient's forearm and this was covered with a glass slide containing a section of the patient's own tumour. After a period of time the slide was removed and the cellular response to the section of tumour could be examined microscopically. Forty per cent of breast cancer patients showed a positive reaction to sections of autologous tumour by this technique,whereas only 10% of this group reacted to sections of normal breast. This reaction was demonstrable 1-2 years after mastectomy and was more frequently present in patients whose lymph nodes showed the features of sinus histiocytosis.The same author

(Black et al.,1978) used both the skin window and leucocyte migration tests as measures of immune response of breast cancer patients to breast tumour cells. A wide variation of results was seen in the patients studied and the conclusion of the study was that the presence of a positive skin window or leukocyte migration test might be of prognostic significance.

One criticism of these studies is that the processing of breast tumour tissue either by preparing soluble membrane extracts,or cutting sections and fixing them on slides may alter the nature of expressed antigens and hence these tests may not be an accurate reflection of what happens in vivo.

Andersen et al.,(1964) described the leukocyte migration test as a measure of cellular immunity. This assay involved measuring the inhibition of leukocyte migration induced by breast cancer cells, this inhibition being a measure of the cell mediated immune response to that antigen. This test was found to be positive in five of eight patients with breast cancers tested with extracts of autologous tumour. None of these patients reacted with extracts of autologous normal breast nor did the leukocytes of any healthy donor react with breast carcinoma extracts.It was concluded that these five patients demonstrated an in-vitro cellular immune response to breast cancer cells.

McCoy et al. (1974) reported the results of the leukocyte migration inhibition assay in 26 breast carcinoma patients using homogenised breast carcinoma extracts. Twenty of these latter patients produced a positive reaction to autologous tumour whereas none of these patients reacted to extracts of either

normal breast or benign breast tumours.There was,however,some crossover reaction in that breast cancer extracts did produce a reaction in a number of normal controls. It was found that the leukocyte inhibition assay compared well with the results of skin hypersensitivity tests in these patients.This study provided further evidence of a cell-mediated immune response to breast cancer cells in some but not all the breast cancer patients studied. Tsang et al. (1980) demonstrated that leukocyte adherence inhibition occurred when peripheral blood lymphocytes of breast cancer patients were treated with autologous tumour extracts. This did not happen using the blood of healthy controls.

These and other studies have demonstrated some evidence of a cell mediated immune response to cancer cell extracts in breast cancer patients.

2.2.2 The Humoral Immune Response to Breast Cancer.

Humphrey et al.,(1974),assessed the reactivity of serum of breast cancer patients with preparations of breast carcinoma cells using both immunodiffusion and complement fixation techniques. Serum was obtained from patients with both benign and malignant breast tumours and healthy controls. A positive reaction using a homogenised breast carcinoma preparation was found in 25% of patients with fibroadenoma, 34% of patients with fibrocystic disease and 46% of patients with carcinoma. None of the 54 healthy controls produced a positive reaction. The breast carcinoma patients who produced a positive reaction were found to react to preparations of a variety of different tumour types as well. Howard et al.(1979) reported the presence of an antibody in pooled AB serum from healthy donors which bound to breast cancer cells as assessed by an immunoperoxidase technique.This serum also bound to breast tissue showing the features of epitheliosis, and the authors suggested that this technique was specific enough to be of value in the histological diagnosis of breast cancer. It was,however, possible that the immunoperoxidase assay used in this study was detecting endogenous immunoglobulin in the breast tumour sections and not bound immunoglobulin from the AB serum. Edynak et al.(1971) described the binding of sera of breast cancer patients to a variety of cultured breast carcinoma cell lines using immunofluorescence. It was found that the sera bound particularly well with a nuclear antigen on one of the breast cancer cell lines. Sera from patients with medullary carcinoma showed the highest degree of binding to the breast carcinoma cell lines.

Normal human sera showed a much lower degree of binding to the breast carcinoma cell lines whilst the positive sera from breast cancer patients did not react with a variety of other tumour and normal cell lines. One criticism of this study was that only cultured cell lines were used as targets and, as will be discussed,the antigenicity of such lines is known to vary with time in culture. It was also possible that the sera was bound to HLA or other non tumour-specific antigens. Wasserman et al.(1975) found a higher incidence of autoantibodies (anti-nuclear, -smooth muscle,-glomerular and anti-mitochondrial) in the sera of 100 breast cancer patients compared with normal controls. This may not, however, represent an immune response to breast cancer but instead be a reflection of increased cell breakdown within the tumour. Nordquist et al.(1977),demonstrated the binding of sera of breast cancer patients to the cultured breast cancer cell line BT-20 and showed,using immunofluorescence, an increased incidence of binding of breast cancer sera compared with sera from healthy controls or patients with benign breast disease. Sheikh et al. (1979) used immunofluorescence to detect binding of patient's sera to sections of their own breast tumours. Sera from 45 out of 104 patients with ductular carcinoma reacted with sections of both their own and other patient's tumours, whereas sera from patients with lobular carcinoma did not react with sections of ductular carcinoma. In addition ,the positive sera were found to react with

other tumour types. Edynak et al.(1972) used similiar techniques to detect binding of the sera of twenty two of twenty four breast cancer patients to sections of autologous tumour,whereas this only occurred in the sera of four of twenty healthy controls.The positive breast cancer sera failed to react with a variety of other cell types. It was concluded in this study that there were anti-breast carcinoma cell antibodies in the serum of many breast cancer patients. Priori et al.(1971) obtained breast carcinoma cells from fresh mastectomy specimens and tested the sera from breast cancer patients, those with benign breast disease, healthy controls and other cancer patients for reactivity against these cells using immunofluorescence techniques. The sera of 30 of 42 breast cancer patients reacted with these cells although there was some cross reactivity with the sera of both benign breast disease patients and also one with an osteosarcoma.

These studies suggested that there were factors in the sera of at least some breast cancer patients which appear to bind to breast carcinoma cells in vitro. This provided some evidence of a host humoral immune response to breast cancer cells. These serum factors, presumably antibodies secreted by B-cells sensitised by tumour associated antigens,have yet to be characterised fully. These studies do have some technical limitations. The use of immunofluorescence made the precise histological localisation of the antibody binding site difficult. In addition this method did

not provide permanent records which would be available for subsequent comparisons. As has been mentioned both the fluorescence and immunoperoxidase techniques used may detect cytophilic immunoglobulin in the tissue sections as well as tumour reactive immunoglobulin from the patients serum.

It is concluded that there is evidence of both a cellular and humoral immune response to breast carcinoma cells in some but not all breast cancer patients.

2.2.3. Clinico-pathological Evidence of an Immune Response to Breast Cancer.

The following studies describe some of the clinico-pathological evidence for a host immune response to breast cancer cells .In some instances attempts have been made to correlate these features of a host response with the course and prognosis of the disease. Black et al.(1953) compared the histology of axillary lymph nodes with survival in 226 patients with breast cancer, in particular looking at the presence of sinus histiocytosis and follicular proliferation. Each of these features was scored on a system of 0-4. It was found that the presence of sinus histiocytosis correlated with a good 5 year survival compared with patients whose nodes did not show this feature. This beneficial effect was seen even in patients with axillary lymph node metastases at time of diagnosis. The same author (Black et al.,1955) described the presence of sinus histiocytosis as being a measure of the host's immune response. Further work was reported comparing the value of sinus histiocytosis, tumour infiltrating lymphocytes and tumour histology as independent prognostic indicators. Although the presence of a high concentration of tumour infiltrating lymphocytes appeared to confer a favourable prognosis only a small number of patients were included in the study. It was suggested that the presence of sinus histiocytosis and perhaps tumour infiltrating lymphocytes were independent prognostic markers for breast cancer. This conclusion might be criticised since approximately 12% of the patients in the study had no record made of their axillary node histology.

Berg (1952), however, was unable to detect any correlation between the presence of sinus histiocytosis in axillary lymph nodes and prognosis in patients treated for breast cancer. The same author (Berg,1971) found it difficult to relate tumour morphology to the presence of an immune response. Hamblin (1968) examined both tumour infiltrating lymphocytes and node histology retrospectively and found that their presence had prognostic significance which appeared to be largely independent of other known prognostic variables. Dire et al.(1963) found that the presence of sinus histiocytosis in the axillary nodes appeared to produce an improved ten year survival. Crile (1967,1968) reported a series comparing radical mastectomy (with axillary node clearance) with simple mastectomy (the axillary nodes being left in situ) for women with no clinically detectable axillary metastases at the time of surgery, and found that this latter group had a somewhat improved prognosis. The authors concluded that uninvolved axillary nodes provided some form of protection against the spread of cancer. This concept is open to criticism since it is difficult to detect axillary metastases by palpation alone. Cutler et al.(1969) examined a group of 100 patients with bilateral palpable lymph nodes in a series of 2000 patients with breast cancer and found that in those patients with no histological evidence of axilllary metastases, those with bilateral palpable nodes had an improved survival compared with those with no palpable nodes. This study suggested that bilateral palpable nodes

hyperplastic and tumour free, represented a beneficial host immune response.Tsakraklides et al,(1974)examined the histology of regional lymph nodes and compared this to survival in patients with breast cancer. A correlation was found between nodes exhibiting lymphocyte predominance and good survival and lymphocyte depletion and poor survival. The authors concluded that lymph node architecture reflected the state of the regional immune response which might affect survival. Anastassiodes et al. (1966) examined the presence of sinus histiocytosis in axillary nodes and described the presence of sinus histiocytosis as a sign of the host's immune response. Tsakraklides et al (1975) examined the relative T and B cell populations in the axillary nodes of mastectomy patients and compared these with the histological appearence of the node and the response of the lymphocytes to mitogen stimulation. A wide variation in all these parameters was observed suggesting a wide range of host immune response to breast carcinoma.

Bloom and Richardson(1970) described the follow up over 20 years of 1411 patients with breast carcinoma of whom 104 had medullary tumours (tumours of high malignancy but with heavy lymphocytic infiltration). This group of patients had a much improved five year survival compared with the rest of the group, and this applied to patients with both positive and negative axillary nodes. It was concluded that medullary cancer, in spite of its high malignancy, had a favourable prognosis because of the immune response it elicited. This study of a large series of patients

over a long (20year) follow up provided important evidence to suggest that a strong immune response may be a favourable prognostic feature. Ownby et al.(1983) measured peripheral blood lymphocytes and eosinophils in breast cancer patients,and found that those patients with a high lymphocyte or eosinophil count had a significantly longer time to reccurrence than those with lowcounts, suggesting that the level of the immune response may affect the time to recurrence.

Eremin et al, (1976) defined the lymphocyte subpopulations in normal human lymph nodes, inflammatory nodes and axillary nodes from mastectomy patients. T and B lymphocyte subpopulations were characterised using a variety of surface markers and rosetting techniques, and the values obtained compared with the findings in the peripheral blood lymphocytes from the same patients. Whilst no difference was found in the blood lymphocytes of the three groups, significant differences were found in the nodes. Regional tumour nodes were found to have an increased percentage of B cells and decreased percentage of T cells compared with normal nodes, although the total lymphocyte counts were elevated. Comparable changes were found in inflammatory nodes and tonsils, both responding presumably to viral and bacterial antigens. In a later report,(Eremin et al.,1980), lymphocyte subpopulations were studied in association with changes of microarchitecture of the node. Increased paracortical thickening and sinus histiocytosis were associated with a relatively increased T-cell count whilst lymphoid follicles were enlarged in those nodes with an increased B- cell count. The changes in the T- and B-cell populations in the

regional tumour nodes were seen to be similiar to those seen in nodes subjected to continuous antigen stimulation ie chronic inflammatory nodes. This provides some evidence that the axillary nodes draining breast cancers are involved in a local immune response and are subjected to repeated persistent antigenic stimulation, although the nature of the antigen is uncertain.

These studies suggest that there is a host immune response to breast cancer. This response appears to involve both cellular and humoral immune mechanisms . The presence and extent of this response has been investigated for its effect on prognosis but no definite relationship between the immune response and prognosis has been established. The presence of an immune response implies that breast carcinoma cells possess antigen(s) which are recognised as foreign by the body's immune surveillance system, thus precipitating the response.The nature and specificity of these antigens remains unclear.

Black et al. (1976) compared the antigenicity of precancerous mastopathy, in situ carcinoma and invasive carcinoma using the skin window technique,and found that a positive response was obtained more regularly and to a greater extent in those patients with preinvasive disease. Cross reactivity between benign and malignant breast tumours was also seen in the work of Humphrey et al. (1974), Priori et al.(1979),and Avis et al.(1976).The same effect has been shown by workers using monoclonal antibodies as will be described in subsequent chapters.

It has been postulated that the tumour associated antigens inducing an immune response might represent de-differentiation antigens which might also be expressed on benign breast tumours. Hollinshead et al.(1974) attempted to isolate the protein fraction of soluble membrane antigens which was unique to breast carcinoma, and succeeded in isolating one protein band which ,as has been described, appeared to have these properties. Kufe et al.(1983) used a variety of monoclonal antibodies to detect breast carcinoma cells at various stages of differentiation and concluded that cancer-associated antigens may only be exposed at certain stages of the cell cycle. Leung et al. (1979) investigated the presence of mammary tumour glycoprotein(MTGP) which was initially obtained from the cytoplasm of 7 breast tumours tested but in none of 54 normal tissues. In a larger series MTGP was found in 76.2% of all cancers and it was postulated that this molecule might represent a specific marker for breast cancer. Rosen et al, (1982) examined the value of pregnancy specific glycoprotein as a marker for breast cancer but found that it had a wide distribution in normal tissues. Old(1981) concluded that whilst the evidence for tumour antigens was extensive and varied it was nevertheless indirect , and tumour specific antigens have yet to be defined directly and specifically.

It is concluded that there is considerable indirect evidence of an immune response to breast cancer,and that breast carcinoma cells do possess tumour-associated antigens not usually expressed by normal differentiated cells.. Axillary lymph nodes have been shown to be similiar to those undergoing persistent antigenic

challenge, and to contain increased populations of B cells. It is proposed that axillary lymph nodes of breast cancer patients do contain B lymphocytes which have been specifically sensitised by tumour associated antigens and that these B cells therefore have the potential to secrete antibody with specificity for breast carcinoma cells . This forms the theoretical basis on which this study into the production of human monoclonal antibodies with reactivity against breast carcinoma cells is based. The following chapter will discuss the theories and techniques of monoclonal antibody production.

2.3. MONOCLONAL ANTIBODIES.

2.3.1. The Initial Experiments

The production of monoclonal antibodies (McAb) of predefined specificity was first described in the now well known work of Kohler and Milstein in the mid 1970's. In their initial studies into the diversity of antibodies, (Kohler et al.,1975) two antibody secreting mouse cell lines were fused together, using inactivated Sendai virus to promote cell fusion. These fused cells ,or hybridomas, were grown in selective medium, and their secreted products investigated. These hybridomas had a chromosome count of just less than that of the sum of the two parent cell lines, indicating that these cells had arisen by fusion of both parent cells. The secreted products of these cell lines and of the original parent cell lines were investigated by immunoelectrophoresis. The hybrids were found to secrete immunoglobulins which were different from those secreted by either parent cell. However, when the secreted immunoglobulins of both parent cells and hybrids were reduced to their constituent light and heavy chains,it was demonstrated that the hybridomas were secreting the same chains found in both parent cells. It was deduced therefore that the new immunoglobulins secreted by the hybridomas arose by different combinations of the various chains secreted by both parent cells. No new immunoglobulin chains were secreted by the hybridomas.

Earlier studies (Milstein et al.,1974) had shown that the V (

(variable) and C (constant) regions of each antibody chain were coded for by one mRNA molecule. It was concluded therefore that fusion of two secreting cell lines produced no scrambling of the V and C regions of each immunoglobulin chain. The production of new immunoglobulins was concluded to be an intracellular event,in that simple mixing of the two parent cell lines resulted in no new antibody formation. Whilst these hybridomas secreted a variety of immunoglobulins, the specificity of these molecules remained unknown.The next important step was to modify this experimental model to secrete antibody of predefined specificity. This was achieved (Kohler et al 1976(a)) by fusing an antibody secreting murine myeloma cell line with a lymphocyte which had been sensitised by a known antigen, in the hope that the resulting hybridoma would secrete antibody against that antigen. A Balb/c mouse was immunised by the injection of sheep red blood cells (SRBC's) which were known to provoke a strong immune response in these mice. After a few days the animals were sacrificed and their spleens removed. The spleen cells were then fused with the mouse myeloma cell line P3-X63 Ag8. The resulting hybridomas secreted immunoglobulin chains different from the myeloma cell line, and it was concluded that the hybrid was secreting immunoglobulin chains coded for by the parent lymphocyte. The chromosome count of these hyridomas was slightly less than the sum of the two parent cells but greater than twice the chromosome number of Balb/c cells indicating that these hybrids did not result from the fusion of two spleen cells.

The resulting hybridomas were cloned on agar and the reactivity of their supernatants was tested for their reactivity to SRBC's by a complement mediated lysis test. Several hybrids were found to secrete antibody with specificity against SRBC's ie antibodies of predefined specificity had been produced by the fusion of a sensitised lymphocyte with a myeloma cell line.

In their second report (Kohler et al 1976 (b)) further work was described which showed that these antibodies of predefined specificity were truly monoclonal. Using similiar techniques to those already described,mice were immunised with either SRBC's or trinitrophenyl (TNP) bound onto fowl immunoglobulin, and the spleen cells of these mice fused with the mouse myeloma line. Supernatants were tested for their reactivity by their ability to lyse 1) SRBC's and 2) SRBC's coated with TNP. Hybridomas positive for lysis were then cloned on agar and these clones were tested for anti-SRBC activity. These supernatants were also characterised by immunoelectrophoresis. It was shown that the positive hybridomas and clones secreted immunoglobulins with different light and heavy chains from those secreted by the parent myeloma cell line,as well as chains from the myeloma cell line . By repeated cloning it was possible to produce a cell line which secreted only the new immunoglobulin chains and none of the myeloma chains. This hybridoma clone secreted antibody with the maximum activity against SRBC's and it was concluded that these clones were producing pure or monoclonal antibodies of predefined specificity.

In summary, the following stages are involved in the production of monoclonal antibodies.

1) A myeloma cell line which transmits the properties of stable growth and antibody secretion in culture.

2) A lymphocyte sensitised by the appropriate antigen and capable of antibody production.

3) The successful fusion of these cells to produce stable hybridomas.

4) The means to detect antibody production by hybridomas and the characterisation of these antibodies.

5) Selective growth of fused cells , so that fused cells will grow in conditions in which the parent cells will die.

6) Cloning of the hybrid cultures to ensure that the antibody produced is truly monoclonal.

The work of Kohler and Milstein represents the foundation of McAb production. The following sections will describe how these techniques have been modified and advanced by other workers in the production of a large variety of monoclonal antibodies.

2.3.2. The Mouse Model.

Myeloma Cell Lines.

Kohler and Milstein used a myeloma cell line derived from a Balb/c mouse for fusing with lymphocytes. This cell line was selected for resistance to 8 azaguanine,thus making it suitable for use in selective medium as will be described. It had,however,the disadvantage of secreting an IgG immunoglobulin chain of its own. Many workers (Kohler et al.,1976(b) , Fazekas 1980 (b),Goding 1980) have commented on the difficulties posed by using a secreting myeloma cell line for McAb production. The resulting hybridomas would have the potential to secrete a number of different immunoglobulin molecules from a variety of combinations of the chains produced by both parent cells. The isolation of the hybridoma(s) secreting immunoglobulin chains derived from only the spleen cell requires considerable effort. There are obvious advantages in using a myeloma cell line which does not secrete immunoglobulin chains of its own but which, when fused with a lymphocyte, will still transmit the properties of perpetual growth and antibody production in culture (Goding 1980). . Such variants of mouse myeloma cell lines have been successfully produced and used for McAb production.

Kearney et al.(1978) produced a non-secreting variant by repeated cloning of a mouse myeloma line and testing the supernatant of each clone for antibody production. These variants were successfully used to produce McAbs by fusion with a splenic B-cell. Continuous culture of these variants did not produce any

revertants throughout the period of testing-ie immunoglobulin production was not resumed by these cells and this mutation appeared to be stable. Hybridomas resulting from this non-secreting variant did secrete immunoglobulin, but none of the chains secreted by the hybrids was similiar to any of the original myeloma chains. Kohler et al.(1976(a)) produced two non secreting variants of the MOPC21 myeloma cell line which were successfully used for McAb production. Such spontaneously occurring mutants were estimated to occur with a frequency of 10^{-3} to 10^{-4}/cell/generation in the myeloma cell culture and these mutants were not seen to revert to antibody production at a later date. Schulman et al(1978) likewise produced a non-secreting murine myeloma cell line variant which was successfully fused to mouse spleen cells to produce antibody secreting hybrids.

In summary non-secreting murine myeloma cell lines have been produced which can be used to form hybridomas which secrete antibody derived only from the spleen cell and this represents a considerable advance in McAb production.

2.3.3.The Spleen Cell

Kohler and Milstein demonstrated by immunoelectrophoresis the production of new immunoglobulin chains by hybridomas of spleen cells and myeloma cells , concluding that this new immunoglobulin must be derived from the spleen cell. It was concluded that the spleen cell was therefore a cell which had been specifically senstised by the appropriate antigen and primed to secrete antibody- ie it had the properties of a B lymphocyte.

2.3.4.Immunisation Schedule.

The immunisation schedules described below outline the regime used to immunise mice with the appropriate antigens prior to sacrifice of the animal and removal of the spleen for use in fusion experiments. Kohler et al,(1976(a), 1976 (b)) reported that there was a considerable variation in the efficiency of hybridoma production. This could be related to the immunisation schedule used and the time interval between the last booster injection and the removal of the spleen. These findings suggest that there is an optimum immunisation schedule for McAb production. Stahli et al(1980) investigated this aspect of McAb production and concluded that succesful fusion required a high frequency of blast cells in the spleen cell preparation which could be achieved by adjusting the immunisation schedule. Andersson et al.(1978), reviewing the evidence that altering the immunisation schedule could improve the rate of hybrid production, attempted to simulate this effect by

prior stimulation in-vitro of the B cell population. The mitogen used was lipopolysaccharide which had been shown to stimulate specifically the B-cell population (Kearney et al.,1975). The rates of hybrid production using mitogen stimulated spleen cells was found to be comparable with that obtained using an optimum immunisation schedule.

It is concluded from these studies that the successful production of McAbs requires lymphocytes which are at the correct stage of differentiation-ie at the blast cell stage when they are actively dividing and are committed to antibody production.

2.3.5.Cell Fusion

The fusion of two mononuclear cells to form a single cell which inherits the properties of both parent cells is central to the work of McAb production.

Fusion between cells of similiar type was shown to occur spontaeously when grown together in in-vitro culture(Littlefield 1964). The rate of fusion,however, between the two mouse fibroblast cell lines used in this study was very low. Efforts were made,therefore,to find agents which would promote cell fusion. Harris et al.(1965) reported the use of Sendai virus, inactivated by ultraviolet light,to promote fusion between human HeLa cells and mouse Ehrlich cells. Yerganian et al.(1966) also was succesful in using inactivated Sendai virus to promote fusion of dwarf hamster cells.

Sendai virus was regarded as a useful fusogen and was successfully used in the production of McAbs by early workers(Kohler et al., 1975,1976(a). There are problems, however, associated with the use of Sendai virus. Its use is limited to laboratories with the facilities to produce inactivated Sendai virus. Its fusion effects tend to be variable and there is always the possibility that virus particles will be incorporated into the hybridomas and hence alter their characteristics. For these reasons Pontecervo (1975) sought to develop a more suitable fusing agent. It had been observed that polyethylene glycol (PEG) promoted fusion between plant fibroblasts . This substance was investigated for its ability to promote fusion between mammalian cells. PEG was successfully demonstrated to promote fusion between mouse and hamster cells and between human fibroblasts and lymphocytes.

PEG has since been confirmed to be a stable and reliable fusogen and has now superceded Sendai virus in the production of McAbs. White et al.(1980) investigated the cellular mechanism of cell fusion using electron microscopy.In these studies it was shown that the' fusogen' promoted fusion between the membranes of the two parent cells with no leakage of contents. Fusion of the nuclei did not occur until both cells underwent the next division. This offers an explanation as to why it is important to have both parental cells actively dividing at the time of cell fusion.

2.3.6.Selective Growth of Hybridomas.

It is important that, when cell fusions are performed, the hybridomas will grow in culture medium in which both parental cell lines will die. It has been estimated that cell fusion occurs approximately once for every 10^6 parent cells used (Kohler et al 1976(a), Fazekas et al,1980(a)). Therefore after fusion, there will be only a few hybridomas mixed in with several million unfused parent cells, and clearly these hybrids would be impossible to isolate if all the parental cells also grew in the culture medium. Two systems of hybrid selection have been described and these will now be discussed.

a) Selective Drug Resistance.

This method depends upon the fact that cells selected for resistance to certain drugs lack specific enzymes which are essential for cell growth.If the two parental cells are selected so that they lack complementary enzymes then neither cell line will survive in culture but hybridomas of these cells will survive as they will inherit enzymes from both parents. Littlefield (1964) described resistance to two such enzymes in a cell fusion involving two fibroblasts. One parental cell line was selected for resistance to 8 azaguanine . These cells were shown to lack the enzyme Guanylic acid -ionosinic acid pyrophosphorylase. The second cell line was selected for resistance to 5 bromodeoxyuridine, and was shown to lack a thymidine kinase. It was shown that neither of these cells would survive in culture medium containing

hypoxanthine, aminopterin and thymidine (HAT). By mixing cultures of the two cell lines and growing this mixture in selective medium, it was found that some colonies of cells survived. These cells were shown to contain approximately twice the DNA,RNA and chromosome content of the parent cells. These cells were also shown to contain both the enzymes thymidine kinase and guanylic acid pyrophosphorylase. It was concluded that these cells represented fused cells which had survived by inheriting enzymes from both parent cells. Reading (1982) described this method in more detail.Cells selected for resistance to 8 azaguanine lack a functional hypoxanthine phosphoribosyltransferase(which is the same enzyme as guanylic acid pyrophosphorylase) and cells selected for resistance to 5 bromodeoxyuridine lack a thymidine kinase. Both of these cells are unable therefore to utilise the "salvage" pathways for purine and pyrimidine synthesis although they can still manufacture these substances de novo. The addition of aminopterin, a folate antagonist which inhibits dihydrofolate reductase, blocks this de novo synthesis. Cells grown in HAT containing medium will fail to multiply and will die. The fused cells however inherit these enzymes from both parents and are able to utilise the hypoxanthine and thymidine in the selective medium to produce purines and pyrimidines by the "salvage" pathway and will survive and grow.

b)Irreversible Biochemical Inhibitors.

Wright (1978) proposed this alternative method of cell selection, in which cells were treated with different biochemical inhibitors to stop their growth. If two cell lines are treated with different

complementary biochemical inhibitors then neither will be able to survive in cell culture,whereas fused cells by inheriting molecules from both parents will be able to survive. A wide variety of inhibitors was suggested by the authors and experience of using two of these was described- iodoacetamide and diethylpyrocarbonate. Whilst these inhibitors were successful in allowing selective growth of hybrids, this system had a number of disadvantages which were noted by the author.This system was unpredictable and difficult to use, and the incorporation of these inhibitors might alter the characteristics of the cells. The system does have the obvious advantage of not requiring selective medium. Littman et al (1983) used this system to produce human-human McAbs by fusing peripheral blood lymphocytes with a variant of the GM1500 human myeloma cell line,using diethylpyrocarbonate. It was concluded that the use of selective drug resistance and HAT medium was more efficient in terms of hybrid production.

The system of selective drug resistance has been most widely accepted and has been widely employed in McAb production.

2.3.7. Feeder Cells.

Fazekas et al.,(1980(a) was interested to discern why the cell fusion technique of Kohler and Milstein produced such variable results. It was concluded by the author from his experiments that the chief indicator of a successful fusion was the presence in the culture tray of active macrophages, these macrophages being derived from the spleen cell population. This phenomenon was investigated by obtaining mouse peritoneal macrophages and adding these to half of the wells in the fusion experiment.The addition of macrophages in this manner was found to improve markedly the rate of hybrid production. The same good results were obtained by adding rat or guinea pig macrophages to mouse hybridomas. The same author(Fazekas 1980(b)) postulated that macrophages were required to remove the cell debris that accumulated as parental cells die off. These products,if left,may inhibit hybridoma growth. Also,the macrophages may produce and secrete substances which promote cell growth. Reading(1982) emphasised the importance of"feeder "cells for successful hybridoma production and pointed out that the addition of feeder cells decreased the number of medium changes required and hence decreased the risk of contamination.

Various cell types have been successfully used as feeder cells-eg mouse peritoneal macrophages(Fazekas et al.,1980(a), Edwards et al., 1982,Schulman et al.,1978), irradiated human fibroblasts (Kennett et al.,1978),and mouse thymus cells(Andersson et al.,1978,Lernhardt et al.,1978,Schlom et al.,1980).

Astaldi (1980,1981) pointed out how little was known of the mechanism by which feeder cells act. The efficiency of mouse peritoneal macrophages as feeder cells was compared with the supernatant from a culture of human endothelial cells (HECS) obtained from a piece of umbilical vein.This supernatant was found to be at least as effective as macrophages in improving hybridoma production, and attempts were made to isolate the effective component of this supernatant. This was thought to be a molecule of molecular weight 750,000 which bound onto the hybridoma cells.

It is possible that feeder cells act either by phagocytosis of cell debris and/or the provision of growth substances which stimulate hybrid growth.

2.3.8. Human Monoclonal Antibodies.

Since the pioneering work of Kohler and Milstein ,the technique of immunising mice with the appropriate antigen and using the spleen cells of these animals for McAb production has been widely used for the preparation of McAbs. McAbs have been raised to a wide variety of antigens of various types including molecules eg Trinitrophenol, (Kohler et al.,1976(a)), virus particles such as influenza (Fazekas et al.,1980(a)), cell membranes (Greene et al., 1980) and whole cells (Fazekas et al.,1980(a)). Such antibodies have found a wide variety of uses in many branches of medical science,and the production of these antibodies has attracted considerable financial investment. The use of mouse-mouse hybridomas does have some disadvantages however, if used in the clinical situation . These are as follows.

Firstly, if a mouse is immunised with human tumour cells (whole or extracts), the mouse is likely to be sensitised to both a large number of species-specific as well as tumour specific antigens (Milstein 1982, Kaplan et al.,1982). As has been discussed, the precise nature of tumour-specific antigens is unclear and it is impossible to immunise the mouse with a pure preparation of tumour-associated antigen. The resulting hybridomas are therefore likely to have a wide range of cross reactivity with normal human tissues and a great deal of cell cloning will be necessary to isolate a clone producing a tumour specific antibody (Burtin et al 1982).

Ashall et al.,(1982) produced a McAb by immunising mice with extracts of a human laryngeal cell carcinoma and the resulting Cal antibody was originally thought to be specific only for cancer cells. When this antibody was further investigated it was also found to react with a range of normal tissues as well. Stepleweski (1980) used both melanoma and colonic carcinoma cells as immunogens and found that only a few of the resulting MABs reacted with the appropriate cell line selectively.There was considerable cross reactivity with many of the McAbs with normal human tissues. Minna et al.,(1981) encountered similiar problems attempting to produce McAbs against lung cancer cells, as did Brown(1982).

Secondly,antibodies produced by mouse/mouse hybridomas will secrete mouse immunoglobulins and there is a significant risk that these will produce hypersensitvity reactions if administered to patients repeatedly (Marx,1982, Bron, 1985). Miller(1981) noted a mild hypersensitivity reaction after repeated injections of a murine McAb to a patient with T cell leukaemia. Larson et al., (1983) discovered the presence of anti-mouse antibodies in the serum of patients who had been given murine McAbs.In two patients a second injection of murine McAb rapidly disappeared from the circulation and this was associated with a large rise in the level of anti-mouse immunoglobulin in their peripheral blood. Thus even if adverse hypersensitvity reactions are avoided there is

the possibility that the usefulness of these antibodies will be severely limited by the formation of anti-mouse antibodies in the host individual.

One method of overcoming these problems has been to try to produce human/mouse hybridomas by fusing a sensitised human lymphocyte with a mouse nonproducer myeloma cell line.It was hoped that these hybrids would secrete human McAbs and that they would be more specific in their activity as the parent lymphocyte was immunised in vivo. Whilst the feasibility of this technique has been demonstrated (Schlom et al.,1980,Sikora et al.,1981),it is associated with various problems. These hybrids tend to be unstable and to preferentially lose human chromosomes (Olsson et al.,1980,Sikora et al.,1982(c)). This method requires early and frequent cloning of all hybrids to produce a stable hybrid. It is possible that these hybrids selectively retain chromosome 14 (responsible for the human immunoglobulin heavy chain) as suggested by Croce et al.,(1979,1980(b)). Gigliotti et al.,(1984) produced mouse-human MAbs against tetanus toxoid, diphtheria toxin and the capsular antigen of Haemophilus influenzae,but found that most hybrids lost their ability to secrete immunoglobulin .

Many workers are agreed that the ideal solution to these problems would be the development of antibody secreting human-human hybridomas, the development of which will now be discussed. Bloom et al.,(1974) first demonstrated the feasibility of fusing together two human lymphocytes. These cells were fused using

inactivated Sendai virus and were grown in selective medium. The fused cells retained a stable chromosome number and secreted immunoglobulins derived from both parent lymphocytes.Both parent cells contained chromosome markers and both of these markers appeared in the fused cells confirming that fusion of the two different cells had occurred.

The first two reports of the production of human/human McAbs of predefined specificity were made almost simultaneously. Olsson et al.,(1980) described the successful fusion of human spleen cells (taken from patients with Hodgkins disease undergoing splenectomy as part of a staging laparotomy) with an 8 azaguanine resistant variant of the human myeloma cell line U266. These patients were immunised prior to fusion with dinitrochlorobenzene (DNCB). Five hybrids were obtained from three fusion experiments and these secreted antibody with activity against dinitrophenol. Croce et al.,(1980(a)) fused peripheral blood lymphocytes from a patient with subacute sclerosing panencephalitis with a human myeloma cell line, and obtained hybrids which secreted antibodies with activity against measles virus antigen.

Following these two reports several groups of workers have reported the production of human McAbs . Shoenfield et al.(1982) prepared autoantibodies by fusing the peripheral blood lymphocytes from patients with autoimmune diseases with a human myeloma cell line. Sikora et al.(1983(a)) fused lymphocytes from a variety of tumours with the human myeloma cell line LICR/LON/HMy2 in an

endeavour to produce anti tumour antibodies. The same author reported on the successful attempt to produce autologous McAbs with some activity against human glioma cells (Sikora 1982(b)).Littman et al. (1983) fused peripheral blood lymphocytes from a patient suffering from systemic lupus erythematosus with a variant of the GM1500 cell line in an attempt to produce monoclonal autoantibodies.

The production of human-human McAbs has proven to be more difficult than the corresponding mouse McAbs. These problems include the low rate of hybrid production (Sikora et al.,1982(c)),the small amounts of immunoglobulin secreted by these hybrids and the difficulties of collecting and separating the lymphocytes which had been sensitised in vivo to the appropriate antigen.Various aspects of the fusion system have therefore been investigated in an attempt to overcome these difficulties.

The human myeloma lines which have been used so far have two disadvantages. Firstly they produce hybrids which secrete low amounts of immunoglobulin. Olsson et al.,(1980) demonstrated immunoglobulin secretion of only 3-11ug/day from hybrid cultures using the U266 line. Edwards et al.,(1982) described immunoglobulin secretion of 0.1-3ug/day from hybrids derived from the LICR/LON/Hmy2 line.

Secondly these myeloma lines secrete immunoglobulin of their own which complicates the analysis of hybrid secretion. For example the U266 line secretes an IgE (Olsson et al., 1980) whilst the LICR/LON/Hmy2 secretes an IgG (Edwards et al.,1982).The desirability of having a nonsecretor human myeloma line for the production of human McAbs has been stated by Marx (1982) who commented on the fact that "Cancer Institute officials hope to be able to budget

$0.5million to remedy this deficiency"(ie the absence of a nonsecretor myeloma).

2.3.9. Epstein Barr Virus Transformation

An alternative approach to cell fusion in the production of human McAbs has been proposed. This involves the transformation of sensitised lymphocytes with Epstein-Barr virus (EBV). EBV transforms B lymphocytes causing them to undergo blast transformation,divide and secrete antibody.EBV is readily obtained from a transformed marmoset line which secretes the active virus (Miller et al.,1973) and this technique has been used to produce human McAbs. Slaughter et al.(1978) used EBV to transform lymphocytes from patients with rheumatoid diease to produce monoclonal rheumatoid factor. Steinitz et al.(1979) transformed blood lymphocytes from patients with high naturally occurring anti nitrosonitrophenol(NNP) activity, and produced antibodies with activity against NNP. Watson,D.B. et al.(1983) attempted to produce anti-melanoma antibodies by EBV transformation of tumour infiltrating lymphocytes. Rosen et al.(1977) measured the increase in IgM and IgG secretion following EBV transformation of normal human and foetal lymphocytes. Steinitz et al.(1980) produced monoclonal rheumatoid factor by the EBV transformation of lymphocytes from patients with rheumatoid disease. Kozbor et al(1982) pointed out the extremely low rate of immunoglobulin production obtained by EBV transformed cells per se and attempted to combine EBV transformation with cell fusion.Lymphocytes were first transformed with EBV and then fused with an oubain resistant variant of the myeloma cell line GM 1500. The potential advantages of this will be discussed in a later chapter.

2.3.10. Monoclonal Antibodies and Breast Cancer.

This chapter will discuss studies already reported which have attempted to produce McAbs with activity against human breast carcinoma cells.

Schlom et al.(1980), Wunderlich et al.(1981), and Teramoto et al. (1982) fused axillary node lymphocytes of breast cancer patients with a murine non-producer myeloma cell line using mouse thymocytes as feeder cells. Supernatants of the resulting hybrids were screened for the presence of human immunoglobulin using a radioimmunoassay and these antibodies tested for activity against breast carcinoma cells by an immunoperoxidase technique. A total of 301 hybrids grew from 1460 wells using lymphocytes from 16 patients. Fifty one hybrids (17%) secreted human IgG or IgM but only 23 of these remained stable in culture.Of these latter hybrids one was selected , cloned and the antibody further investigated.This antibody stained approximately 50% of cancer cells in sections of the patients own breast tumour. The percentage of cells stained and the intensity of staining varied widely throughout the tumour. The antibody did not react with surrounding normal breast tissue or stroma, but did react with cancer cells in axillary node metastases. The antibody reacted with a number of other breast cancers, and to a lesser extent both normal breast tissue and benign breast tumours from different patients. There was some degree of cross reactivity with other tumour types e.g.bronchial alveolar cell carcinoma and medullary carcinoma of thyroid, but none with a selection of normal tissues.

This study demonstrated the feasibility of using axillary lymphocytes to produce McAbs with activity against breast cancer cells. It is of interest that the staining reaction varied widely throughout the tumour-this may be an artefact or may represent variability of antigen expression within the tumour . The antibody did show some activity against juxtatumoural normal breast cells in some patients and also against some benign breast tumours This suggests that these cells are in a stage of de-differentiation and are beginning to express the tumour-associated antigen or alternatively indicates that the antibody is not specific for tumour cells. The cross reaction with other tumour types may indicate a cross reactivity with other antigens having a similiar epitope or it may be that the antigen recognised by the McAb is not specific to the breast cancers but is shared by other tumours. This study also indicated the instability of most mouse-human hybrids although the one hybrid investigated had survived more than 300 days in culture.

Ashall et al , (1982) and McGee et al (1982) described the production of the Cal antibody, obtained by immunising mice with extracts of a human laryngeal cell carcinoma. This antibody was seen to bind to a wide variety of human tumour types using an immunoperoxidase technique. Initial work suggested that this antibody bound quite specifically to breast carcinoma cells. Subsequent investigation by different workers,however,has

failed to confirm this. While it was originally suggested that this antibody recognised a tumour-specific antigen subsequent work showed that this antibody also bound to a variety of normal tissues.

Yuan et al.(1982) and Hendler et al (1981) produced a murine McAb by immunising mice with the cultured human breast carcinoma cell line ZR75-1. Of the twenty antibodies produced one was shown by immunofluorescence to bind onto the ZR75-1 cell line and not to a variety of normal human cells. This antibody showed positive binding to 3 out of 6 other cultured human breast carcinoma cell lines. When tested against sections of breast tumours positive binding to tumour cells was detected in 54% of tissue sections although the degree and intensity of binding varied considerably. The antibody also bound cells in 84% of fibradenomas and 82% of fibrocystic disease. It was suggested by the authors that this antibody only reacts with well differentiated,oestrogen receptor positive cancers. This antibody may recognise antigens which are present in benign tumours and well differentiated carcinomas but these antigens may disappear as the tumour becomes less well differentiated. The technique of using cultured breast carcinoma cells as immunogen is open to criticism. Breast carcinoma cells are very difficult to grow in culture and the few established cultured cell lines available have been distributed widely amongst laboratories with the risk of contamination with viruses and other cells. It has been demonstrated that these cell lines can alter their characteristics markedly with repeated passages in culture and therefore these lines may not represent the most appropriate immunogen.(Hand et al.,1983).

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Colcher et al.(1981) used a membrane extract of cultured breast carcinoma cell lines (MCF7, BT20,ZR75-1)as immunogens to produce a murine MAb which showed some activity against the parent cell lines. The antibody also showed binding to a number of other tumour cell lines of different origins but did not react with cell lines derived from normal tissues. This study did not include a record of the reactivity of this antibody to sections of breast tumours which limits its usefulness. It lends some support to the suggestion by Ashall et al (1982) that many tumour types share common antigens although the presence of such common antigens remains to be confirmed. Greene et al (1980) used a purified form of the extranuclear oestrogen receptor protein from the MCF7 breast cancer cell line as immunogen and produced an antibody with a variable activity against cells which possessed receptors for oestrogen. Foster et al.(1982) used the human milk fat globule membrane as immunogen in the production of murine McAbs. Four antibodies were obtained which bound,to a variable extent and degree of intensity, to both normal and malignant breast tissue.This variable degree of uptake suggested a heterogeneity of antigen expression throughout the tumour cell population.The binding pattern of these McAbs to breast cancers did not seem to correlate with the degree of differentiation of the tumour which led the authors to suggest that these antibodies may provide different markers from tumour histology and therefore be useful probes for investigating the functional status of breast cancers. Taylor-Papidimitrou et al.(1981) likewise used the human milk fat globule membrane as immunogen and obtained antibodies which reacted with both normal and malignant breast cell

lines.These McAbs also showed some binding to pharyngeal and colonic cancer cell lines. Arklie et al . (1981) further investigated the specificity of this McAb against a variety of tissue sections of normal and lactating breast , benign and malignant breast tumours and found that the antibody bound well to both lactating breast and some carcinomas. Some degree of binding was also seen with a number of benign tumours suggesting that this antibody recognised tissue-specific rather than tumour-specific antigens. In the case of tumours,the antibody stained well differentiated tumours better than poorly differentiated ones and lobular tumours better than intraduct tumours. Some binding to other tumour types e.g. adenocarcinoma of lung and ovary was also detected. Papsidero et al.(1983) used the cultured breast cancer cell lines MCF7 and SK-BR3 as immunogens. Of the resulting murine McAbs two were selected which bound to the parent lines . When tested against sections of breast tumours, neither of these antibodies was found to bind selectively to breast carcinoma cells. This study demonstrated the difficulties of using cultured cell lines as immunogens. Menard et al,(1983) was likewise unable to produce a specific anti-breast cancer McAb using MCF7 as immunogen. Adams et al,(1983) prepared a murine McAb by immunising mice with a soluble extract of the MCF 7 cell line. This McAb was found to bind to a cytosol preparation of the MCF 7 cell line and it was suggested that this McAb may provide a means of measuring the oestrogen receptor status of breast cancers. King et al, (1984) described a panel of murine McAbs which were raised against oestrogen receptor protein, and which identified nuclear

oestrophilin in sections of breast tumours ,using an immunoperoxidase technique. Heterogeneity of McAb binding was noted throughout the tumour cell populations. Hand et al.(1983) investigated the binding patterns of four murine McAbs on 39 specimens of breast carcinomas and found that the pattern of staining varied widely both within and between tumours suggesting that breast carcinomas do possess a wide variety of antigenic heterogeneity. Sikora et al. (1983(a)) fused lymphocytes from axillary nodes of mastectomy patients with the human myeloma line LICR/LON/Hmy2. Binding of the resulting McAbs to breast carcinoma cell lines was weak and no binding of these McAbs to sections of actual breast tumours was demonstrated by immunohistological staining. Capone et al,(1983) produced murine McAbs by immunising mice with cells derived from breast tumour specimens,and these McAbs were found to bind to breast tumour cells and a variety of other epithelial tumour types,but not to a variety of normal human tissues. Breast tumours were then xenografted into nude mice. Subsequent injection of this McAb was found to dramatically decrease the size of the xenografted tumour causing considerable tumour necrosis. By radiolabelling this McAb specific targeting of the McAb to the xenografted tumour was demonstrated. However no attempt was made to xenograft normal human tissues , or other types of tumour to determine whether this effect was specific to breast tumours or not. Peterson et al.(1983) demonstrated a high degree of variability of antigen expression in populations of breast carcinoma cells by using a McAb to a single cell surface component of the breast epithelial cell.

In conclusion a wide variety of McAbs of both mouse and human origin have beeen developed but none of these has been shown to possess absolute specificty for breast carcinoma cells. These studies do demonstrate 1) the feasibility of using axillary node lymphocytes of breast cancer patients to produce anti-breast cancer McAbs, 2) some potential uses for anti-breast cancer McAbs (such as the measurement of oestrogen receptor status),and 3) the fact that there appears to be a wide variability of antigen expression within and between breast tumours, with some cross reactivity with benign breast disease. It is also possible that antigens on breast cancer cells may be shared by a variety of other tumour types.

2.3.11. Clinical Applications of Monoclonal Antibodies.

The potential clinical application of McAbs has been investigated in both experimental and clinical situations. This chapter will discuss three main areas in which McAbs may be of value in the management of malignant disease. These are 1) the investigation of tumour antigens and tumour behaviour 2)the detection of occult tumour deposits and 3)the selective killing of tumour cells.

2.3.12. The Investigation of Tumour Antigens and Their Relationship to Tumour Behaviour.

As has been discussed there is evidence that breast tumours (and other tumours) possess tumour specific or tumour associated antigens although the precise nature of these antigens is as yet unknown. McAbs may have a role in characterising these antigens and defining their distribution throughout the tumour. Such work may provide important markers for cancer cells thus giving more information about the behaviour of the tumour which may be of prognostic significance.

Foster et al.(1982) and Burchell et al.(1983) described the distribution of the anti-milk fat globule membrane antibody in a variety of breast tumours and concluded that the antigen recognised by this antibody was independent of tumour histology, oestrogen receptor status or other known variable. It was concluded that this antibody may provide information of prognostic significance. Similiar heterogeneity of antigen expression has been demonstrated using McAbs in melanoma cell lines (Bruggen et al 1983, Suter et al, 1983), colorectal carcinomas (Daar et al., 1983) and gastric carcinoma (Hockey et al., 1984).

Leinard et al. (1985) reviewed the heterogenous expression of oestrogen receptor in breast tumours and metastases using McAbs. This heterogeneity occurred within individual tumours and was seen to alter in the same tumour after a period of time and after the introduction of hormonal therapy, suggesting that the expression of oestrogen receptor was constantly changing. This supported the theory that malignant cells are continually mutating and altering their antigenic characteristics . McAbs have been of value in defining the heterogeneity of antigen expression within tumour cell populations. This heterogeneity, which has been described as being independent of the cell cycle (Edwards 1985) , is of importance since it may limit the clinical potential for McAbs. Edwards (1985) discussed the nature of the heterogeneity of tumour cell antigens and postulated that this may be due to alterations of the carbohydrate portionof the membrane glycoproteins on cell membranes. He postulated that it may be possible to raise McAbs against the constant protein fraction of these glycoproteins although this has not yet been done. Baldwin (1985) discussed the problem of using McAb /cytotoxic drug conjugates to treat cancer and concluded that a "cocktail" of McAbs may well be required to identify all tumour antigens. Neville et al.(1985) discussed the implications of tumour cell heterogeneity, and likewise concluded that there is a need for a panel of McAbs to treat cancer adequately. McAbs have been used in a diagnostic role when routine histology has failed to provide an accurate diagnosis. Kemshead et al.(1983(a)) described the use of a panel of McAbs in the diagnosis of a series of small round cell tumours in children. In

each case McAbs provided the accurate diagnosis where routine histological examination of the tumour specimen could not define the precise diagnosis. Debus et al.(1984)described the staining pattern of a number of murine McAbs raised against various types of cytokeratins to a variety of tumour sections of different origin. Different staining patterns were discerned depending upon the type of tumour section used -e.g. squamous carcinomas stained differently from adenocarcinomas. It was proposed that these antibodies may be of value in distinguishing tumour types and discerning their histological origin. Ghosh et al.(1983) described the use of McAbs to detect tumour cells in histologically negative aspirates from pleural and peritoneal effusions. Gatter et al. (1982) used a panel of McAbs to distinguish anaplastic carcinoma from high grade lymphoma. A number of cases were described in which these McAbs provided an accurate diagnosis where normal histological assessment was unreliable. These studies illustrate some of the ways in which McAbs are being used to define tumour antigens and characterise more precisely different tumours.

The Detection of Occult Tumour Deposits.

By labelling antibodies with radioactive tracers it may be possible to use tumour-specific antibodies in the detection of both primary and secondary tumours.Labelling of antibodies can either be achieved internally by growth in medium containing H^3 leucine ,or by conjugating the McAb with a radioactive tracer such as I^{131}.It is important 1)that the means of labelling the antibody does not affect the binding pattern of the antibody,and 2)that the tracer is securely bound to the antibody,has a short half life and is easily detected by external scanning. For these reasons labelling is usually performed with radioactive iodine. Pressman (1980) reviewed the techniques available for labelling and concluded that labelling with I^{131} was an appropriate technique. Cuello et al.(1982) internally labelled McAbs by growth in 3H lysine and found that the labelled antibody had the same binding pattern as the unlabelled antibody concluding that internal labelling did not affect the activity of the antibody. Ghose et al.(1975) reported that the conjugation of I^{131} to a polyclonal antibody did not affect the activity of that antibody.

Radiolabelled antibodies have been used in both animal and human studies to detect tumour deposits. Ballou et al.(1979) used an I^{125} labelled mouse McAb to detect a mouse teratocarcinoma successfully by external scintigraphy.

Xenografts of human tumours in immunosuppressed mice have been used to detect the binding of anti-human tumour McAbs.This technique does have the disadvantage of failing to demonstrate whether the McAb recognises a tumour-associatedantigen or a species-specific antigen. Herlyn et al.(1983) conjugated mouse McAbs,raised against human colonic carcinoma,with radioactive iodine and then injected this antibody into mice containing xenograft tumours of human colon.The latter were obtained by the intraperitoneal injection of human colon carcinoma cells. The animals were sacrificed after injection of the antibody and the activity measured in various tissue types. It was concluded that a high proportion of the antibody was bound to the xenografted tumour,especially if only the F(ab)2 fragment of the antibody was used. Similar results were obtained by Mach et al.(1974) using a polyclonal anti-CEA antibody conjugated with I^{131}. Moshakis (1981(a),1981(b))iodinated a mouse McAb with activity against human teratoma cells using I^{125} and used this antibody to detect tumour binding in a xenografted mouse. A high degree of antibody binding to tumour,compared with other tissues,was noted using autoradiography of the tissue samples. Warenius et al.(1981) attempted to target an anti HLA mouse McAb to a human tumour xenografted to a mouse. The antibody appeared however to have a higher uptake in the mouse liver than the tumour. It was deduced that this was due to circulating tumour antigen forming immune complexes in the blood, these complexes being subsequently concentrated in the liver. This may present a major problem in using radiolabelled

antibodies to detect tumours -i.e. the injected McAb may fail to reach the tumour. This may occur because of circulating antigens in the blood which will bind the McAb or else the McAb, being a foreign protein, may itself become bound to circulating host antibodies. Colcher et al.(1983) used an I^{125} iodinated McAb to detect a human breast cancer xenografted onto a mouse. The best uptake was found if only the F(ab)2 fragment of the antibody was used.

The following studies describe some of the human work performed with radiolabelled McAbs. Sears et al.(1981,1982(a)) described the ex-vivo perfusion of the human colon with radiolabelled murine McAb with activity against colorectal carcinoma cells. It was suggested that this may provide a useful model to assess the in vivo activity of such antibodies. Such a model, however, failed to take into account any binding of the antibody whilst in the circulation, nor dids it provide information about the binding of the antibody to other tissues in vivo i.e. it failed to define the in-vivo specificity and homing properties of the antibody. The results of the above studies were not promising. Three out of the six colons perfused showed preferential binding to the tumour, two showed binding to normal and tumour tissue,whilst one failed to show any binding of antibody at all. Smedley et al.(1983) injected a rat McAb raised against human colon carcinoma cells radiolabelled with I^{131} into 27 patients. Scintigrams were performed 48 hours after injection of antibody and tumour uptake in areas of known disease was recognised in 13 out of 16 patients with colorectal cancer and 3 out of 4 patients with breast cancer. The quality of the imaging however was poor.

Brown (1985) injected ^{125}I-conjugated McAbs into mice xenografted with human soft tissue sarcoma and found that the maximum uptake of McAb by the xenograft occurred at 7 days. Sears et al.(1982(b)) reported a series of four patients who received injections of mouse McAbs. Anti-mouse immunoglobulin antibodies were detected in three of these patients but no adverse reaction was noted clinically. One patient, on the other hand, received multiple injections and developed a mild hypersensitivity reaction after the 4th injection. Dykes et al.(1980) injected polyclonal sheep anti CEA antibody labelled with I^{131} into a series of patients with large bowel cancer.Four out of five primary tumour sites and 8 out of 11 secondary deposits were identified on the subsequent scans, which helped to confirm the potential feasibility of using radiolabelled antibodies as diagnostic agents. Larson et al. (1983)injected a murine McAb with anti melanoma activity labelled with I^{131} into six melanoma patients. This technique successfully identified 22 out of 25 lesions larger than 1.5cm in diameter. Whilst no adverse clinical reactions were noted, anti mouse immunoglobulin antibodies were subsequently identified in the serum of three patients. Berche et al.(1982) injected murine monoclonal anti CEA antibody labelled with I^{131} into patients with colorectal carcinoma. External scintigraphy successfully identified 16 of 17 sites of known tumour, 5 of these deposits having volumes of less than $10cm^3$. Mach et al.(1983) used an I^{131} labelled murine McAb with activity against colon carcinoma cells to detect by tomoscintigraphy 34 out of 63 known deposits of

colon carcinoma in 52 patients.

Farrands et al ,(1983) used a murine McAb labelled with I^{131} to detect an osteosarcoma by rectilinear scintigraphy.In conclusion,these studies,which are all preliminary in nature involving small groups of patients,have shown the feasibility of using radiolabelled antibodies to detect sites of tumour deposits. The sensitivity of the techniques needed to be improved, however, since these antibodies identified only large tumour deposits currently detected by existing techniques. Neville et al. (1985) described the conjugation of ^{111}Indium to McAbs and concluded that this was a preferable isotope to ^{125}I for use in the immunodetection of breast tumours.

2.3.14. Tumour Therapy.

The use of McAbs as a means of selectively killing cancer cells has the potential of being one of the major advances in cancer therapy. McAbs have been looked upon as the potential "magic bullet". The following paragraphs will describe some of the work that has been reported investigating the potential use of antibodies as selective cancer cell cytotoxic agents.

Antibodies may induce the death of the target cell in the presence of complement. This has been demonstrated in vivo. Berche et al.(1982) described the administration of a murine McAb with activity against a T-cell differentiation antigen in a patient with adult T-cell leukaemia. Immunofluorescence studies did show strong antibody binding to the T-cells. Following the administration of the antibody on two occasions the white cell count fell dramatically but then rose over the ensuing 24 hours. Also, a mild hypersensitivity reaction was noted 5 days after treatment. Thus while the antibody produced a marked effect on the leukaemia cell population, this effect was transient. Nadler et al.(1980) injected a mouse McAb with activity against leukaemia cells (AB89 antigen) into a leukaemia patient and showed that the resultant effects,as measured by serial blood counts,was both small and transient. This McAb had been shown to produce complement-mediated lysis of these cells in vitro. Bast et al.(1983) described the selective killing of leukaemia cells, diluted X100 with normal human bone marrow cells,by a

murine McAb in the presence of complement. Watson,J.V. et al. (1983) described a chamber which could be implanted subcutaneously in which hybridomas secreting antibody could grow. The hybridomas could not pass out of the chamber into the tissues whereas the McAb could freely disperse. This chamber was used to grow autologous human hybridomas secreting antibody with activity against glioma cells in a patient with a glioma. No beneficial effect was observed however in the patient's clinical course. Miller et al, (1982) described the administration of murine anti-B cell McAb to a patient with advanced B-cell leukaemia. After 8 doses of the McAb over a four week period the patient remained in remission for six months. Buckman et al, (1982) used a murine McAb to eliminate cancer cells from bone marrow in-vitro,in the presence of complement. Gore et al,(1983) detected killing of the MDA-MB cultured breast carcinoma cell line (using a Cr^{51} release assay) by the administration in vitro of an anti-MDA McAb in the presence of killer lymphocytes derived from human bone marrow. Kaszubowski et al. (1984) produced a murine McAb against colonic carcinoma cells which were found to be toxic to these cells in the presence of human complement.

Whilst the effect of administration of McAbs on their own has found some success, they may be of great value for use as carrier molecules to deliver cytotoxic agents selectively to cancer cells. Ghose et al, (1978) commented that early work conjugating conventional cytotoxic drugs to polyclonal antibodies had been disappointing. A study was reported in which ricin A chains were conjugated to a McAb which was

then shown to be toxic to mouse macrophages and rat Kupffer cells in vitro. This demonstrated that antibody could take the place of the B chain of ricin to bind the A chain to cells. However this conjugate was found to be less toxic than free ricin.

Levy et al (1975) provided further evidence of the feasibility of these techniques by conjugating daunomycin with polyclonal anti-tumour antibodies and showing that these conjugates were toxic to tumour cells in vitro. Gilliland et al.(1980) described the use of ricin A chain conjugated to mouse McAb with activity against colorectal carcinoma cells. The activity of this antibody conjugate on a culture of colorectal cancer cells was measured by a C^{14} leucine labelling method which measured the level of protein synthesis in the target cells. The antibody conjugate markedly decreased the protein synthesis of the target cells. It was also found that conjugation of the ricin A chain did not appear to affect the specific binding patterns of the McAb. Seto et al. (1982) demonstrated the selective in-vitro killing of MM 46 cells by murine anti-MM46 McAb conjugated with ricin A chain. The same effect was then demonstrated in-vivo by developing xenografts of these cells within the peritoneal cavity of mice. Tumour regression was demonstrated in these animals following the administration of anti-MM46 McAb/ricin A chain conjugate. Colombatti et al.(1983) demonstrated selective killing of Thy 1-2 bearing cells in-vitro by anti-Thyl-2 McAb conjugated either to gelanin or ricin A chain.

Krolick et al, (1980) conjugated ricin A chains to antibodies against IgG and IgM and tested these conjugates for their ability to kill B-lymphocytes. The ricin A chains were also conjugated with I^{125}.The degree of toxin binding to the cells was then measured by a radioimmunoassay, and the effect of the toxin by measuring protein synthesis. The antibody conjugates were tested against a variety of normal and malignant B -lymphocytes and it was found that the conjugates bound only to those cells bearing the appropriate surface marker. Youle et al, (1977) conjugated McAbs with activity against the Thy 1-2 antigen with whole molecules of ricin. The antibody conjugate was found to bind selectively to THY1-2 antigen bearing cells only in the presence of lactose. The B chain of ricin is said to bind to cells via galactose bearing receptors on the cell surface, this binding being blocked by lactose. If no lactose was present the antibody conjugate bound nonselectively to a variety of cells, this presumably because the ricin was attached to the cell by the B chain rather that the McAb. Thorpe et al(1978) investigated the use of polyclonal anti-lymphocytic serum. From this the IgG was separated out and conjugated with diphtheria toxin. The toxicity of this conjugate was tested against the CLA4 cell line (a lymphoblastoid cell line) using radiolabelled leucine to measure protein synthesis. Toxicity of the conjugate was found to be greater than the toxin alone or a simple non-conjugate mixture of toxin and antibody. Seon (1984) used a McAb/ricin A chain conjugate to demonstrate selective killing of T-leukaemia cells

in vitro, and postulated that this antibody/toxin conjugate may be of value in clearing bone marrow of tumour cells in vitro. Blythman et al. (1981) described the conjugation of ricin A chains with anti THY1 - 2 McAb. The activity of this conjugate was measured in mice innoculated with THY1 - 2 bearing leukaemia cells. Using this system most untreated tumour bearing animals were dead within 39 days whereas the survival of animals treated with the conjugate was significantly increased. Animals treated with a simple mixture of A chain and McAb behaved in the same way as those treated with A chain alone indicating that the antibody required to be conjugated with the McAb to exert a beneficial effect. Kishida et al. (1983) conjugated a murine McAb with ricin A chain and described selective killing of leukaemia cells in culture using this conjugate. In addition the lives of nude mice xenografted with leukaemia cells were significantly prolonged after the administration of this conjugate.

The above studies have demonstrated the feasibility of using toxin - antibody conjugates to selectively kill tumour cells. The potential clinical benefit of using antibody/toxin conjugates will depend upon the uniformity of antigen expression within the tumour cell population and the ability of enough antibody to bind to each cell to allow the absorption of an adequate amount of toxin (Bagshawe 1983). More recently, conventional cytotoxic drugs have been conjugated to McAbs successfully by using an intermediate carrier molecule. Rowland et al. (1985) conjugated Vindesine to a murine McAb and produced regression of xenograft tumours (melanoma, osteogenic sarcoma, and colorectal carcinoma)

in mice, using the appropriate McAb. Baldwin (1985(a)) described the conjugation of methotrexate with a murine McAb using dextran as the carrier molecule. This conjugate was used to treat a xenografted osteosarcoma in a mouse , and was found to be more effective in producing tumour regression than administration of methotrexate alone. The same author (Baldwin 1985(b)) described the conjugation of other cytotoxic agents (Adriamycin, Vincristine and Cyclophosphamide) to McAbs . These conjugates were shown to be effective in killing tumour cells in vitro.

SECTION 3.

MATERIALS AND METHODS.

3.1. Cell Preparation.

3.1.1. Lymphocytes.

Axillary lymph nodes were obtained from patients undergoing surgery for carcinoma of the breast. All of these patients had operable T1-T2 tumours with no detectable evidence of distant metastases at the time of surgery on the basis of screening investigations performed which included a haematological / biochemical profile (full blood count, E.S.R., serum urea and elecrolytes,liver function tests, calcium, phosphate, albumin), chest x-ray, isotope bone and liver ultra-sound scan. A record was made of the patients age,tumour size ,type of operation, lymph node histology and oestrogen receptor status from all patients whose nodes were used in this study. Lymph nodes were obtained following three types of operation-1) simple mastectomy followed by axillary node sample (removal of four axillary nodes) 2) mastectomy followed by clearance of all axillary contents and 3) lumpectomy followed by lower axillary sample or clearance (ie clearance up to the lower border of the axillary vein). Further information about these patients is contained in appendix 2 .

Following the surgical removal of the axillary contents the nodes were immediately dissected out of the axillary fat in an aseptic manner in the ante-room of the operating theatre. Nodes which were macroscopically uninvolved by tumour deposit were then selected out. In cases where only a few nodes were obtained these nodes were bisected, half being retained for use in this study, the other half being submitted for histological evaluation

In cases where many (more than ten) nodes were obtained one node was set apart for use in this study.

Nodes were placed in sterile normal (N) saline and transported to the laboratory within 1-2 hours.

On arrival in the laboratory lymphocytes were separated from the nodes in the tissue culture hood as described by Eremin et al (1976). A 5 ml syringe with a 21g needle was filled with TCM-A/2%FCS. The node was placed in a sterile petri dish and punctured with the needle. The node could then gently ballooned with the culture medium. This process was repeated several times at different sites on the node and by so doing the lymphocytes were readily washed out through the puncture holes. The lymphocyte suspension in the petri dish was then aspirated and centrifuged at 250g for 5 minutes. The lymphocyte pellet was resuspended in TCM-A/2% FCS and re-centrifuged. This washing process was repeated twice more and then the cells were suspended in TCM-A/10%FCS. A drop of cell suspension was then placed in a counting chamber and the number of morphologically viable lymphocytes counted.The cell suspension was then stored at $4^{o}C$ until used in a fusion experiment. This was always performed within 24 hours of obtaining the node (the timing of the fusion experiment being dependent on the availability of a suitable HMy2 culture) , unless the lymphocytes were prestimulated with pokeweed mitogen(PWM).

3.1.2. The Myeloma Cell Line.

The myeloma cell line used in this study, LICR/LON/HMy2 ,hereafter designated HMy2, was kindly donated to the laboratory by the Ludvig Institute for Cancer Research in Cambridge.

The HMy2 line was cultured in TCM-B/10%FCS. Stock cultures of this line were maintained in 100ml glass bottles at 37°C. On the day prior to fusion the myeloma culture was subcultured by splitting the stock culture into three bottles and adding fresh TCM-B/10%FCS to each bottle. One of these bottles was kept as a stock culture, the other two being cultured overnight and used the following day for the fusion experiment when the maximum number of cells would be in the log phase of growth.

3.2. Cell Fusion.

Cell fusion was accomplished using polyethylene glycol(PEG) as fusogen (Pontecervo,1975). The fusion procedure is based on that described by Sikora et al. (1983(a)). Cell fusions and all subsequent feeding of culture plates was performed in an Envair microbiological safety cabinet,class II.(plate 1).

For a standard fusion $5x10^7$ lymphocytes were fused with $5x10^6$ HMy2 cells. The two cell suspensions were centrifuged separately at 400g for 5 min. and resuspended in 15 ml of TCM-B with no added serum. They were then recentrifuged and this washing process repeated three times. The two cell suspensions were then mixed in a 50ml conical tube (Corning) and centrifuged at 400g for 5 min.. After pouring of the medium the tube was left to stand for 5 min. to allow any medium on the sides of the tube to settle to the bottom. This collection of medium was then gently aspirated with a fine bore pipette to ensure that the cell pellet was completely dry.The cell pellet was broken up by gentle flicking of the tube and 0.5 ml of PEG solution A (see appendix 1) was added dropwise. The cell suspension was gently mixed with the PEG by gently stirring with the tip of a pasteur pipette for 20 sec. followed by gentle rocking of the tube. One minute after the addition of PEG sol.A, 0.5ml of PEG solution B was added dropwise and the tube gently rocked for 3-4 min.. Four ml. of TCM-B/10%FCS were then added dropwise following which the cell suspension was gently aspirated into a wide bore 25ml. glass pipette and distributed evenly over the 2ml. wells of two 24 well tissue culture plates

(Nunc). An additional 1.0ml of TCM-B/10%FCS was added to each well and the plates, labelled, were placed in a 5%CO_2,95% humidity incubator (Leec) at 37°c.

3.2.1. Management of Cultures.

On the day following fusion 1.5ml of medium was removed from each well using a pasteur pipette attached to a continuous suction device and replaced with 1.5 ml of fresh selective medium (TCM-B/10%FCS with either HAT(Hypoxanthine,Aminopterin and Thymidine) or HAzT(Hypoxanthine,Azaserine and Thymidine))(see Appendix 1 for details). This feeding process was continued every 2 days for the first week after fusion and weekly thereafter(or earlier if the medium began to turn yellow before one week).Hybrid growth was recognised first by microscopic and then by macroscopic examination of the wells of the plate. Microscopically hybrids appeared as clumps of growing cells in a field of cell debris. Shortly after this clumps of growing cells could be seen macroscopically in these wells. The wells were fed as required until the concentration of the hybridoma cells was sufficient to turn the colour of fresh medium yellow within 24 hours. At this stage the hybridoma culture was divided equally among 3 new wells in a clean plate as well as the original well and these were fed until they too contained actively growing clumps of hybrid cells.At this stage attempts were made to grow the hybridomas in bulk culture in a 50ml tissue culture flask (Nunc) as described by Edwards et al (1982). Whilst TCM-B was initially used in the flask cultures it was difficult to prevent the medium turning alkaline in the flask even when the flask was gassed with CO_2 . The

culture medium was therefore changed from TCM-B in the culture plate to TCM-A in the flask culture. Hybrids were found to tolerate this change in medium .

Cells from three of the four wells containing hybridoma cells were transferred to the flask which contained 5 ml of TCM-A/10%FCS with the appropriate selective medium and after gassing with CO_2 the flask was incubated at 37°c in the ordinary incubator. The flask cultures of hybrids were fed and divided as necessary. The supernatants of these cultures , containing any immunoglobulin secreted by the hybrids were collected whenever the culture medium turned yellow. These supernatants were stored at 4°C in a solution of 10mM Hepes buffer /0.1% sodium azide to preserve the pH of the supernatant and to prevent the denaturing of any antibody present (Sikora et al.,(1983(a)).

Hybrids were maintained in full selective medium for the first 15 passages in flask culture and then the Aminopterin or Azaserine was omitted,leaving the cells in medium with added Hypoxanthine and Thymidine for a further 10 passages. After this the cells were grown in ordinary TCM-A/10%FCS with no additives.

3.3. Pokeweed Mitogen Stimulation.

Pokeweed mitogen (PWM) is a plant lectin which has been shown to selectively stimulate the B-cell population of lymphocytes although this effect has been shown to depend on the presence of T-cells (Keightly et al.,(1976). It has been postulated that prestimulation of lymphocytes prior to fusion with PWM will increase the efficiency of hybrid production as more lymphocytes will be actively dividing at the time of fusion.(Warenius et al., 1983). Preliminary experiments were therefore conducted to measure the effects of PWM stimulation on axillary node lymphocytes.

PWM. Preliminary Experiments

Experiments to measure the effects of PWM stimulation were performed in both a "macro" and a "micro" system - both of these being identical except for the number of cells involved. These two methods were used because of the requirements of the different techniques used to measure the effects of PWM stimulation as will be described.

Whilst human AB serum is often preferred for mitogen experiments because FCS possesses some weak mitogenic effects on its own, FCS was used throughout in these experiments to closely simulate the actual treatment of lymphocytes prior to fusion.

In the macro system axillary node lymphocytes were centrifuged at 400g for 5 min. and resuspended in TCM-A/10%FCS at a concentration of $2x10^6$ cells/ml. PWM was added to 4 ml of this suspension to give final PWM concentrations of 1,4,and 16ug/ml. In addition control tubes with no added PWM were included in each group of experiments. The cultures were fed every second day by

the addition of 2 ml of fresh medium. In the micro system washed axillary node lymphocytes were suspended in TCM-A/10%FCS to a concentration of 1.25×10^6 cells/ml. Using a pasteur pipette two 40ul drops of the cell suspension were dispensed into each of the 96 wells of a microtitre plate. An additional 40ul of TCM-A/10%FCS was then added to each well ,followed by 40ul of PWM to give final PWM concentrations of 2,4,8,16,and 32 ug/ml. Control wells with no added PWM were included in each plate. The plate was then coverd in Transpaseal and incubated at 37°C.

Different techniques were employed to measure the effects of PWM stimulation, these being summarised by Nespoli et al.(1978).

1) Cell Counts.

Lymphocytes were counted both before and after PWM stimulation in a slide chamber using phase contrast microscopy. Counts were made of the total and large blast cell count by two independent observers and the mean count recorded. It was thought that cells stimulated by PWM would progress to the stage of blast cell formation and hence the effects of PWM stimulation could be measured by a relative increase in the number of large cells morphologically resembling blast cells.

2) Measurement of Surface Immunoglobulin (SIg) Bearing Cells.

Attempts were made to determine whether PWM stimulation of B-lymphocytes could be determined by measuring the increase in the percentage of surface immunoglobulin (SIg) bearing cells. As lymphocytes are stimulated by PWM the B-cells pass through the blast cell stage to form plasma cells. Since PWM may stimulate T-lymphoblasts,cell counts alone are not reliable and SIg bearing cells were therefore enumerated.

The measurement of SIg-bearing cells was performed by two techniques, a) an immunoperoxidase technique and b) the formation of anti-human Fab rosettes. The details of these two techniques are described in detail in subsequent paragraphs.

In brief, the immunoperoxidase technique used rabbit anti human IgG and IgM antibodies (Dako) to determine SIg-bearing cells on cytocentrifuge preparations of PWM-stimulated lymphocytes, Sig bearing cells cells showing up as a red-brown colour compared with the blue counterstain. Results were expressed by counting 200 cells and expressing the number of SIg cells as a percentage (see section 3.8.2. for details of immunoperoxidase method).

Anti-human Fab rosettes were prepared as described in section 3.6.2. and again counts were made of 200 cells.

3) Tritiated Thymidine Uptake.

This assay measured the uptake of thymidine into cell cultures. As the uptake of thymidine is directly related to the level of cell division this assay can provide a direct measure of the level of cell division produced by PWM stimulation. This assay has the advantages of being sensitive and of measuring directly the effect of PWM which is most relevant to cell fusion experiments -ie the amount of cell division produced in the lymphocyte culture.

After a period of incubation in PWM, in the micro system, the wells of the microtitre plates were pulsed with ^{3}H thymidine (Amersham),(37 BQ or 1 u Ci) by adding 25ul of the stock solution to each well. The wells were again covered with Transpaseal and

incubated for 18 hours at 37°C. Following this the cells were harvested using a Titertek cell harvester onto a glass fibre disc. The disc was dried and then placed in a container to which was added scintillation fluid. The scintillation fluid used was 4g P.P.O.(2,5 diphenyloxazole (Kochlight))in one litre of Toluene (Fisons). The activity of each container was measured in a Beta counter (Nuclear,Chicago,Mark 1). Counts were made for 2 minutes for each well and a mean count of each group of triplicate wells, with standard deviations, recorded.

3.4. Feeder Cells

The beneficial effects of feeder cells in the production of hybridomas was investigated by the use of two types of feeder cells. Since this study concentrated on the production of human-human hybridomas it was decided to use only human feeder cells to prevent any overgrowth of cells from a different species. The feeder cells investigated were peripheral blood monocytes and thymocytes.

3.4.1. Monocytes

Monocytes were obtained from the peripheral blood of healthy women age 18-70 years who were not known to be suffering from any inflammatory or neoplastic disease. Samples were all obtained with the informed consent of these women. 30 ml of blood was removed into a syringe containg 30 units of preservative free heparin . The heparinised blood was then diluted 1:3 in phosphate buffered saline (PBS) and layered onto Ficoll-Hypaque (Specific gravity 1.007) (30ml diluted blood onto 10 ml Ficoll Hypaque in a siliconised glass tube). These tubes were then centrifuged at 400g for 40 min. at 20°C (centrifuge MSE Mistral GL), thus separating

the lymphocyte/monocyte population at the interface of the Ficoll-Hypaque from the granulocytes and erythrocytes at the bottom of the tube, as described by Boyum (1968). The lymphocyte/monocyte layer was carefully pipetted from the interface and washed twice in TCM-A/2%FCS at 400g for 10 min. and used as described in section 4.5.1..

3.4.2. Thymocytes.

Thymocytes were obtained from samples of thymus gland from children under the age of 5 undergoing open heart surgery at the Royal Hospital for Sick Children, Edinburgh. A part of the thymus gland is routinely removed in such children to allow exposure of the heart. Samples of thymus were placed in sterile saline in the theatre and transported directly to the laboratory where thymocytes were removed in the same way that lymphocytes were removed from the lymph nodes. By this means large numbers of thymocytes were obtained and these were washed four times by suspension in TCM-A/2%FCS and centrifugation at 400g for 5 min..At the time of cell fusion thymocytes were added directly to each of the wells of the plate ,10^5 cells/well immediately after the cell fusion suspension had been distributed over the wells.

3.5. Cell Numbers.

The effect of varying the cell input into the fusion system was investigated as follows. In the standard experiment $5x10^7$ lymphocytes were fused with $5x10^6$ HMy2 cells, a lymphocyte/myeloma cell ratio of 10:1. The effect of reducing this ratio to 5:1 and 1:1 was investigated by increasing the number of HMy2 cells used for the fusion. Three 50ml conical tubes were set up each containing $2.5x10^7$ lymphocytes from the same lymph node. To these were added respectively $2.5x10^6$, $5.0x10^6$ and $2.5x10^7$ HMy2 cells.These three mixtures were then fused under identical conditions and each tube distributed over one 24 well plate. All plates were fed with HAT medium,with no feeder cells and no prestimulation of lymphocytes with PWM.

3.6. Characterisation of the Hybridomas.

The hybridomas were characterised by measuring their chromosome count and by characterising their surface markers using a rosetting technique.

3.6.1. Karyotyping.

Cells were karyotyped by arresting the cells in metaphase using colchicine and then swelling the nuclei with hypotonic KCL as described by Moorhead et al (1969).

The technique is summarised as follows.

1) On the day prior to karyotyping the hybridoma culture was fed with fresh medium and incubated overnight to ensure that a large number of cells would be dividing.

2) Colchicine was added to the cell suspension to give a final concentration of 7.5×10^{-6} M and the cells incubated for a further 4 hours at 37°C.

3) The cells were then centrifuged at 400g for 5 minutes , washed twice in TCM-B with no added serum and resuspended in 0.5 ml of this medium.

4) 0.5 ml of 0.075 M KCl was added slowly and the cells carefully mixed. The time was noted at the start of this additiion. A further 3.5 ml of KCl was added slowly and the tube left to stand for 3 minutes.

5) The cells were then centrifuged at 200g for 6 minutes and resuspended in 0.25 ml of KCl. Total time in KCl was 10 minutes.

6) 4 ml of fresh fixative (Acetic acid/Methanol 1:3) was added slowly and clumps of cells gently broken up. The tube was left to stand for a further 15 minutes. The cells were then centrifuged

at 100 g for 6 minutes and the above fixation process repeated.

7)The cells were resuspended in 1 ml of fresh fixative,and a drop of cell suspension was dropped from a height of 6 inches onto a cleaned chilled wet glass slide.Excess liquid was blotted off and the slide gently dried in air. The slides were stained with Giemsa for one hour then examined microscopically. Chromosomes were counted from at least 3 different nuclei and the mean of these counts recorded.

3.6.2. Surface markers.

The surface markers of hybridomas were evaluated by a sheep red blood cell (SRBC) rosetting reaction, to demonstrate that these hybrids were B-cells and not T-cells. This was to confirm that the hybrids had arisen by fusion of a HMy2 cell with a B and not a T lymphocyte.

T-cells form spontaneous rosettes with SRBC's. B-cells are identified by using SRBC's coated with the antibody sheep anti-human Fab to detect surface immunoglobulin.(Eremin et al 1976).

a) T-Cell Rosette Indicators.

1)Sheep red blood cells (Clun Forrest, less than one week old)were washed in PBS at 1600g for 5 minutes.

2) The SRBC's were then suspended in PBS to give a 10% solution.

3) To 50ul of 10% SRBC's was added 500ul of neuraminidase (at 0.01units/ml) in PBS.

4) The SRBC solution was then incubated at 37^{o}C for 20 minutes.

5)Cells were then washed in PBS four times and resuspended in 50 ul of TCM-A/10%FCS and 450ul of FCS absorbed against SRBC'S.

6) The indicators were then stored at 4°C until use.

b) Sheep anti Human Fab (B-Cell) Rosettes.

Indicators for these rosettes were made by conjugating polyclonal anti human Fab with SRBC's using chromic chloride by an identical method to that described in section 3.7.1.

c) Rosettes.

To make rosettes 100ul of the appropriate indicator cells were added to an LP3 tube followed by 100ul of the hybridoma suspension. The rosettes were then incubated,T rosettes for one hour at room temperature, B rosettes at 4°c for 30 minutes. Following this the tubes were gently rotated to resuspend the cells . One drop of cell suspension was then placed on either end of a siliconised glass slide followed by one drop of stain(Toluidine Blue) and covered with a siliconised cover slip.Rosettes were read by counting 200 cells which had stained blue, and recording the number which had formed rosettes(defined as 4 or more attached red cells).

3.7. Characterisation of Supernatants.

3.7.1. Estimation of Immunoglobulin

The presence of antibody in these supernatants was detected by an agglutination reaction involving SRBC's coated with an appropriate antibody using chromic chloride. The first step in this assay was to couple the antibody to the SRBC and this was performed as follows.

1) The SRBC's were washed six times in PBS (400g for 5 minutes). The cells were then suspended in PBS to make a 10% suspension.

2) The appropriate volume of red cell suspension to be coupled was then mixed with an equal volume of 0.25% trypsin(pH 7.0, at 37°C)and left in a 37°C water bath for 30 minutes , with occasional inversion. After 30 minutes the cell suspension was centrifuged and washed twice in PBS at 400g for 5 minutes.

3)The cells were resuspended in trypsin inhibitor in an equal volume to that of the packed red cells.This mixture was left to stand for 10 minutes at room temperature then centrifuged and washed twice in 0.9% NaCl. The cells were then ready for coupling.

4) Coupling was performed in Falcon 2054 tubes. 25ul of a 1% solution of the antibody to be coupled (rabbit anti human IgG and rabbit anti human IgM (Dako Denmark, code nos.A090 and A091) were added to the tube followed by 25ul of packed trypsin treated red cells. 50 ul of chromic chloride (0.2% pH 5.0) was then added slowly whilst the tube was continually shaken. After 30 seconds

the tube was capped and rotated slowly for 60 minutes at room temperature on a Matburn rotator .

The cells were then washed in PBS 3 times (400g for 5 minutes)and resuspended to a final concentration of 1% in PBS. The two cell indicators, one coupled with rabbit anti human IgG and the other with rabbit anti human IgM were then ready for use in the agglutination reaction.

Agglutination Reaction

For each batch of cell indicators produced, control agglutination reactions were performed to test the adequacy of the coupling. Thus each set of indicators was tested against sheep serum (a negative control to test for non-specific agglutination of the cell indicators) and normal human serum (a positive control). Agglutination tests were performed on microtitre plates (Nunc). First, one drop of each supernatant or control serum was added to each well in serial dilutions starting at neat and then in 1 in 4 dilutions serially along the first 11 wells of the row. One drop of the cell indicator was then added to each well and the plates left to stand for $1\frac{1}{2}$-2 hours. The agglutination reaction was then recorded on a scale of 0-3+.

3.7.2. Measurement of the Concentration of Antibody.

The concentration of IgG or IgM in the supernatant was measured as described by Watson,D.B. et al. (1983) using the above agglutination technique. Normal human serum was obtained and the levels of IgG and IgM were measured by the Dept. of Clinical Chemistry, Royal Infirmary, Edinburgh. This normal serum was then agglutinated in serial dilutions until no further agglutination was observed. Since agglutination requires an adequate amount

of antibody,it is presumed that the dilution at which no further agglutination occurs represents the dilution at which there is insufficient antibody present. The hybrid supernatant was then agglutinated in serial dilutions until it too failed to produce an agglutination reaction.The concentration of antibody in the supernatant could then be calculated as follows.

$\frac{Sc}{Sd} = \frac{Cc}{Cd}$ where Sc = supernatant concentration

Sd =supernatant dilution

Cc = control concentration

Cd = control dilution.

3.8. Screening of Supernatants for Reactivity to Breast Carcinoma Cells.

3.8.1.The Radio-immuno Assay (fig. 1)

This assay was used in an attempt to detect binding of antibody in the supernatants of hybridomas to a variety of cultured cell lines, derived from both breast carcinoma cells and other cell types. It is based on the techniques described by Sikora et al(1983(b)). The cell lines used were MCF-7 and MDA_MB (breast cancer cell lines) , CLA4 (a lymhoblastoid cell line) and DET6 (a HeLa subline)(see appendix 1 for details of these cell lines).These cells were cultered as monolayers (apart from CLA4 which grew as a suspension culture) until a sufficient cell density was obtained . The cells were then removed from the flasks by trypsinisation and washed by centrifugation in PBS five times. The cells were then resuspended at a concentration of $5x10^6$ cells/ml. The assay was performed using flexible polyvinyl u-well microtitre plates. These were pretreated with poly-L-lysine(0.1% solution in PBS),50ul/well for one hour at room temperature. The poly-L-lysine was then removed and 50 ul of the appropriate cell suspension added per well. The cells were allowed to settle at 4°C for one hour and then the plates were carefully immersed in 0.25% glutaraldehyde in PBS, avoiding air bubbles,and left for 5 minutes at 4°C. This process caused adherence of the cells to the wells of the plate as described by Stocker et al. (1979). Some authors (Posner et al.,1982, Lansdorp et al,1980) suggest that the use of glutaraldehyde in this way increases the degree of non-specific

binding in this assay.

The glutaraldehyde was then flicked out and the plate immersed in PBS for 5 minutes at 4°C and then for 5 minutes at room temperature,to wash out excess glutaraldehyde. The plates were then dried on absorbent paper.

100ul of blocking buffer(PBS+0.1% sodium azide +1% bovine plasma albumin)was then added to each well and left for 60 minutes at 37°C. This was to block any non specific binding sites on the surface of the plastic of the wells to which antibodies might adhere.

50ul of the test supernatant or appropriate control was then added to each well and incubated for 45 minutes at room temperature. The supernatant was removed by flicking the plate and the wells washed 5 times with PBS to remove all free supernatant.

The plates were then dried on paper and 50 ul of mouse anti human Ig Kappa or Lambda chains (Bethseda Resaearch Labs cat. nos.31040 and 31030) at a 1/5000 dilution was added to the wells and incubated for 45 minutes at room temperature. The plates were again washed five times in PBS and dried.

50ul of I^{125} conjugated rabbit anti mouse antibody(Dako) was then added per well and incubated at room temperature for 45 minutes. The plates were finally washed five times with PBS and dried. The plates were then cut up into individual wells and the bound radioactivity measured in a gamma counter (Wallac). Each cell type was set up in triplicate with each of the hybridoma or control supernatants and the results calculated as the mean of the counts of each of the three triplicate wells.

Iodination of rabbit anti mouse immunoglobulin used in the radioimmunoassay.

This procedure was performed in the fume cupboard in the grade B laboratory in the Dept. of Surgery. Prior to performing this procedure I attended a course of lectures on the correct management of radioactive materials organised by the University of Edinburgh. I acknowledge with thanks the supervision and advice given by Mr.J.Ashby during this procedure.

Materials used.

1) 1mCi (37mBq) of ^{125}I Sodium Iodide (Cat.no. IMS 30, Amersham International).

2) Chloramine T 1mg/ml in distilled water.

3) Saturated tyrosine solution (Millipore filtered).

4) 1% bromophenol blue in distilled water.

5) Sephadex G50 column.

6) Phosphate buffered saline

7) PBS + 1% bovine serum albumin + 0.1% Sodium Azide.

PROCEDURE.

1) Add 50ul of antibody to the vial if ^{125}I and immediately add 10ul of Chloramine T.

2) Close vial, mix and leave for 90 secs.

3) Add 50ul of saturated tyrosine.

4) Add 10ul of bromophenol blue.

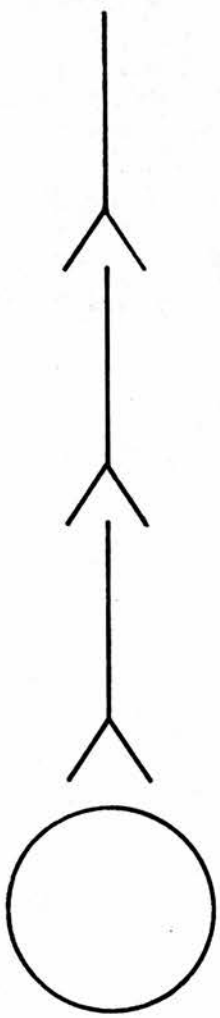
5) Load into Sephadex column.

6) Elute 200ul fractions into LP3 tubes with PBS.

7) Pool the fractions with the highest counts on the gamma counter and make up to 5 ml with PBS/BSA/Sodium azide.

FIGURE 1.

THE RADIOIMMUNOASSAY.



RABBIT ANTI MOUSE IMMUNOGLOBULIN ANTIBODY CONJUGATES WITH RADIOACTIVE IODINE

MOUSE ANTI HUMAN KAPPA (LAMBDA) CHAIN ANTIBODY

HYBRIDOMA SUPERNATANT

BREAST CANCER TARGET CELL

3.8.2. The Immunoperoxidase Technique.

This technique was employed as well as the radio-immuno assay to detect the binding of MAbs to breast carcinoma cells. This assay is designed to detect sites of binding of McAbs to target cells using the enzyme horseradish peroxidase.Horse-radish peroxidase can then be detected by the addition of an appropriate reagent which will turn red-brown thus allowing sites of McAb binding to be detected microscopically.This assay has the advantage that it can detect binding of MAbs to sections of breast tumours as well as cultured cell lines, thus giving an estimate of the possible in-vivo site of reactivity of the antibody. (Naiem 1982).There are several methods by which the peroxidase technique can be performed. The enzyme horseradish peroxidase can be conjugated directly onto the McAb thus giving the "direct" assay. Alternatively, the enzyme may be bound onto a polyclonal antibody raised against the enzyme and then this "peroxidase-anti-peroxidase" complex can be bound onto sites of McAb binding by using an appropriate intermediate antibody. This indirect assay, whilst somewhat more complex, is said to increase the sensitivity of the assay many fold (Taylor,1978) and was the method used in this study. A variety of target cells were used which were prepared as follows.

1) Cytocentrifuge preparations were made of both cultured breast cancer cell lines and also of cell suspensions derived from tumour specimens. The former consisted of both MCF7 and MDA-MB (see appx.1).

The latter were prepared by finely mincing fresh breast tumour tissue with a scalpel blade, incubating this minced tissue in collagenase for 16 hours at 37°C and then straining through a muslin filter.(Eremin et al 1982). The resulting cell suspension was then washed twice in TCM-A/10%FCS, incubated for 12 hours at 37°C, then washed and resuspended to a final concentration of 10^6 cells/ml.

In addition cytocentrifuge preparations were also made of tumour cell suspensions in which Fc rosettes(to identify macrophages) had been formed. Fc rosette indicators were produced as follows.

1) Ox rbc's, inagglutinable and less than one week old, were washed x6 in PBS and resuspended in 2% PBS.

2) 500ul of 1/10 rabbit IgG prepared against ox rbc's in PBS was added to 500ul of ox rbc suspension.

3) Cells were then incubated for 45 min. at room temperature.

4) Cells were washed x2 in PBS, resuspended in TCM-A/10%FCS and stored at 4°C. Rosettes were then formed as described in section 3.6.2(c).

These cell suspensions were added to the chambers of the cytocentrifuge (Shandon Cytospin 2)(1 drop/chamber) and centrifuged at 800 rpm for 5 min.. The resulting slides were air-dried and fixed in acetone for 10 min..Prior to use in the peroxidase assay these slides were rehydrated in water at room temperature for 30 min..

2)Paraffin sections of breast tumours were obtained from both the Dept of Clinical Surgery and the Department of Pathology, Edinburgh University. These sections came from patients from whom nodes had also been obtained to perform fusion experiments. The

slides were dewaxed prior to use in this assay by immersion in xylol for 10 min., followed by washing in alcohol in concentrations of 100%,70%,20% and 0% in water.The slides were then treated with trypsin. This was because it has been suggested (Curran et al 1977) that the fixation methods used on paraffin sections may "mask" a variety of antigens .Pretreatment with trypsin was thought to "unmask" these antigens and hence make the assay more sensitive. Slides were therefore washed in 0.1% trypsin (pH 7.6) at 37°C for 15 min..

Immunoperoxidase Staining.

1)Slides were first incubated in methanol/3%H_2O_2 for 30 min. at room temperature to block any endogenous peroxidase activity in the target cells. It has been suggested that false positive background binding in this assay might be due in part to the presence of endogenous peroxidase in the target cells and that this activity might be blocked by incubation in H_2O_2(Heyderman et al 1977).

2) Slides were washed in tris-buffered saline(TBS)for 10 min. at room temperature and then incubated in 20% normal swine serum (NSS) in TBS at room temperature for 20 minutes to block any non specific binding sites on the glass slide. Swine serum was chosen because it would not react with any of the other antibodies used in this assay.It was obtained from the local abbatoir and heat-inactivated for one hour at 56°C prior to use.

3) The slides were rinsed in TBS/2%NSS for 15 min. and then the hybrid supernatant was added to the slide and incubated at room temperature for 30 min. in a moist chamber.

4)Following a 15 min. wash in TBS/2%NSS the second antibody (rabbit anti human IgG(sigma) or IgM(u) (Dako) 1/100 dilution in TBS) was incubated for 30 min. at room temperature.

5)The washing process was repeated and then Swine anti Rabbit immunoglobulin (Dako) was added, again for 30 min..

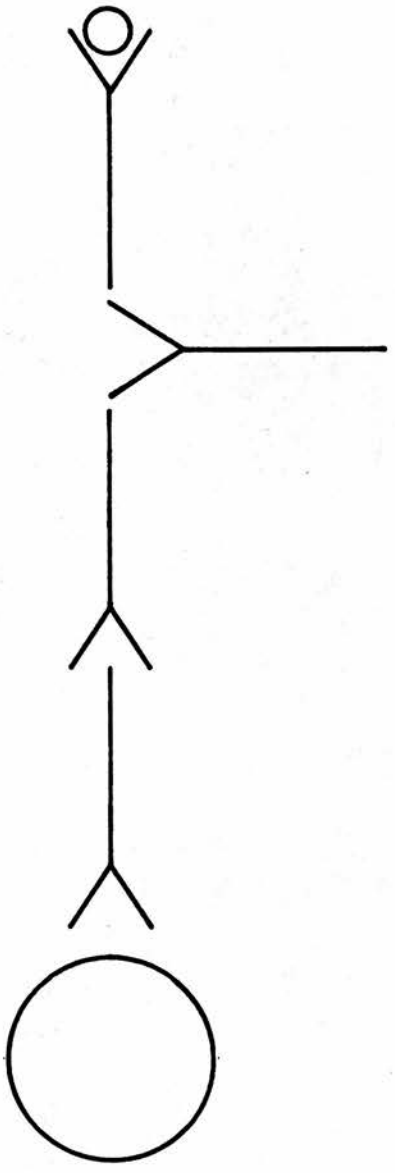
6) Finally the slides were washed and incubated with the peroxidase/antiperoxidase complex(rabbit,Dako) for 30 min. at room temperature.

7)The slides were washed again and the peroxidase stained by the addition of Amino-Ethyl Carbizole (0.5ml diluted in 9.5ml of acetate buffer) to which 2 drops of 30 vol H_2O_2 had been added immediately before use. This stained the peroxidase a red-brown colour and the slide was then counterstained in haemotoxilyn.

This assay is summarised in figure 2.

FIGURE 2.

THE INDIRECT, P.A.P., IMMUNOPEROXIDASE REACTION.



P.A.P.

S.A.R.

RABBIT ANTI HUMAN
IgG (IgM) ANTIBODY

HYBRIDOMA SUPERNATANT

BREAST CANCER TARGET CELL.

3.9. Cloning of Hybridomas.

Hybridoma cultures were subcultured on the afternoon prior to cloning to ensure that most cells were actively growing and dividing by the following day. For cloning, hybridomas were suspended in TCM-A/10% FCS, and cell counts performed. The hybridoma culture was diluted by an appropriate amount to give suspensions of 100,50 and 25 cells/ml. One 25ul drop of each suspension was then added to the wells of a 96 well microtitre plate to give 4,2 and 1 cells per well. Two drops of TCM-A/10% FCS were then dispensd into each well and the plates cultured in the CO_2 incubator (Leec) at 37°C. Culture medium was changed regularly, and the cell cultures observed under inverse phase microscopy. When the cells were seen to be growing in clumps, they were transferred to the 2ml wells of the 24 well tissue culture plates (Nunc) and fed with regular medium changes. Finally the hybridomas were transferred back to the 50ml tissue culture flasks, where they remained until recloning occurred.

SECTION 4.

RESULTS.

4.1. Introduction

The results of the fusion experiments are given in the following paragraphs. Fusion experiments were labelled with the prefix "FE"(Fusion Edinburgh)followed by the number of the experiment. Since most fusion experiments involved more than one plate, the plate number was then added. Finally the wells of the plate were denoted by the grid system on the plate,each well being denoted by a number on the "x"axis and a letter on the "y" axis. Thus a hybrid would be defined by a title such as FE30/4/A2. The lymph nodes used in the experiments were labelled by the prefix T.N.L.E.(Tumour Node Lymphocyte Edinburgh) followed by the appropriate number.

Initial fusion experiments had been performed in the laboratory prior to the commencement of this study, these being labelled FE1 to FE19. Whilst the details of these experiments are not included in this thesis since the work was not done by me, the hybridomas obtained from these experiments were characterised by me and these results are included within this thesis.

4.1.1. Scoring System.

Fusion experiments were scored by recording the number of hybrids obtained per 24 well plate. No time limit was set after fusion at which the plates were scored, because of the widely varying times after fusion at which hybrids appeared. All plates were therefore fed and cultured as described until such time as infection of the plate by moulds ,yeast or bacteria was seen to have occurred at which time the plate was discarded. However an arbitrary minimum time limit was set. All plates had to survive this period in culture before they were included in the results,those which failed to do

so being counted as technical failures. This lower limit was arbitrarily set at 24 days, being the earliest time at which a hybrid appeared (59/3/A3).

4.2. Results- Fusion Experiments.

The results of the fusion experiments performed in this study are described first ,as the introductory group of experiments, then as the overall results of the main group of fusion experiments, and finally as the groups of fusion experiments evaluating the different options employed in an endeavour to improve the rate of hybridoma production.

4.2.1. The Early Experiments . FE 20-30.

Infection.

Prior to the commencement of this study infection of the culture plates by yeast organisms within the first 10 days after fusion was a major problem within the laboratory. The early experiments were therefore used firstly to learn the techniques of cell fusion and cell culturing and secondly to combat the problem of yeast infection. Before any fusions were done, the CO_2 incubator was emptied and thoroughly cleaned and all glassware re-sterilised. It was then planned to investigate the use of Fungizone(Amphotericin B) to combat yeast infection by adding it to the culture medium in varying concentrations. These early experiments are summarised as follows. The plates of FE 20 - 25 were all discarded before day 24, either because of the early development of infection , or because of a technical error during the fusion experiment. FE 26-30 had fungizone added in varying concentrations to each row of wells from 0 to 2.5 ug/ml. In each of these plates, when yeast

infection occurred, it occurred in those wells with either no fungizone (FE 29/1,29/2.29/3,27/1,27/2,27/3,27/4) or else a low concentration of fungizone (1.0 ug/ml, FE 29/4). The rest of these plates succombed to either yeast or mould infection, having survived longer than 24 days in culture.

No hybrids were obtained from any of these plates.

In these varied early experiments ,fungizone was seen to be of value in preventing yeast infection in experiments FE26-29.The only yeast infection occurring in the presence of fungizone was plate FE29/4, in a fungizone concentration of 1.0ug/ml. Since the maximum recommended dose of fungizone is 2.5ug/ml for cell culture work, hybrids obtained from the early fusion experiments were changed into medium containing fungizone at this concentration. This did not appear to affect the growth characteristics of these hybrids at all. In view of these results fungizone was added to all culture medium at a concentration of 2.5ug/ml to combat yeast infection. These early experiments are summarised in table 1.

TABLE 1

SUMMARY OF EARLY FUSION EXPERIMENTS FE20 to FE30.

PLATE	FUNGIZONE CONC.	FUNGIZONE TIME	INFECTING AGENT	TIME DISCARDED {DAYS}
20/1	-	-	Y	15
20/2	-	-	Y	10
20/3	-	-	Y	10
20/4	2.5	12	Y	15
21/1	2.5	4	B	9
21/2	-	-	Y	10
22/1		1	B	5
22/2	-	-	Y	7
23/1-4 }	TECHNICAL			
25/1-4 }	FAILURES.			
26/1	0-2.5	1	Y	59
26/2	0-1.0	1	M	69
26/3	0-2.5	1	M	69
26/4	0-1.0	1	Y	59
27/1	0-2.5	1	Y	20
27/2	0-1.0	1	Y	27
27/3	0-2.5	1	Y	20
27/4	0-1.0	1	Y	19

TABLE 1 {CONTINUED}

PLATE	FUNGIZONE		INFECTING AGENT	TIME
	CONC.	TIME		{DAYS}
28/1	0-2.5	1	B	28
28/2	0-1.0	1	B	28
28/3	0-2.5	1	B	28
28/4	0-1.0	1	B	28
29/1	0-2.5	1	Y	12
29/2	0-1.0	1	Y	12
29/3	0-2.5	1	Y	12
29/4	0-1.0	1	Y	39
30/1	0-2.5	1	B	17
30/2	0-1.0	1	B	17

TOTAL

PLATES 34

FAILURES 24

HYBRIDOMAS 0

(fungizone concentration in ug/ml.)

4.3. Fusion Experiments FE 31-101

4.3.1. Progress in Culture and Incidence of Infection

Table 2 summarises the success of culturing plates of experiments FE 31-101,which form the main part of this study. The average survival time of plates in culture in this group was 47.34 days after fusion, which means that the majority of plates survived well over the 24 day minimum culture period. Each plate was ultimately discarded when infection was seen to have occurred in one or more wells of the plate.

Table 3 summarises the ultimate fate of these plates in terms of infecting organisms. From this it can be seen that the introduction of fungizone (and possibly improved technique)was successful in combating yeast infection. Only 42 plates were infected with yeast organisms in this group at an average 52.78 days after fusion. Table 4 shows the number of failures as a percentage of the total plates for each group of 10 fusion experiments. This demonstrates the impact that improved experience had in the prevention of infection. Infection by yeast and mould organisms appeared to occur in epidemic form within the incubator. In a few days many plates would become infected particularly those adjacent to each other in the incubator and this would be followed by weeks in which virtually no infection occurred. Most bacterial infections occurred shortly after fusion and tended to affect groups of plates from the same experiments, suggesting some form of direct contamination around the time that the experiment was performed.

TABLE 2.

SURVIVAL IN CULTURE OF PLATES OF LATER EXPERIMENTS FE 31-FE101.

TOTAL NUMBER OF PLATES	277
"FAILURES" {INCLUDING 5 PLATES WITH NO RECORD}	54
FAILURE RATE	19.49%
AVERAGE DAYS IN CULTURE {ALL PLATES}	47.34 {RANGE 1-144}
HYBRIDOMAS	25

"Failures" = All plates which failed to survive 24 days in culture.

TABLE 3.

RECORD OF INFECTION FE31-FE101

INFECTING AGENT	NUMBER OF PLATES	AVERAGE DAYS IN CULTURE	RANGE
BACTERIA	17	8.12	1- 32
YEAST	42	52.78	19-121
MOULD	213	49.86	11-144

INFECTION OCCURRING BEFORE 24 DAYS.

INFECTING AGENT	NUMBER
BACTERIA	16 {94.12% OF ALL BACTERIAL INFECTIONS}
YEAST	3 {7.14% OF ALL YEAST INFECTIONS}
MOULD	30 {14.08% OF ALL MOULD INFECTIONS}
TOTAL	49.

The plate was discarded when infection was seen to have occurred in one or more of the wells of the plate. Previous attempts to salvage plates with only one infected well by aspirating the well and irrigating it with alcohol failed to prevent the spread of infection through the plate.

TABLE 4.

Ratio of "Failures"/Total Plates Divided into Groups of Ten Fusion Experiments.

Experiment No.	Total Plates	Failures	Failures/ Total Plates x100
FE 20-30	34	24	70.6%
FE 31-40	42	13	30.9%
FE 41-50	46	20	43.5%
FE 51-60	38	10	26.3%
FE 61-70	29	4	13.8%
FE 71-80	45	3	6.6%
FE 81-90	42	6	14.3%
FE 91-101	34	0	0

TABLE 5.

EFFECT OF RAISING THE MINIMUM DAYS REQUIRED IN CULTURE ON THE RATE OF HYTBRIDOMA PRODUCTION.

MINIMUM DAYS IN CULTURE	PLATES	HYBRIDS	HYBRIDS/ PLATE	HYBRIDS/ WELL	HYBRIDS/ 106 LY.
24+	231	25	0.108	0.004	0.009
30-39	174	25	0.143	0.006	0.011
40-49	122	25	0.204	0.009	0.016
50-59	93	25	0.268	0.011	0.022
60-69	70	25	0.357	0.015	0.029
70+	43	25	0.581	0.024	0.047

SUMMARY OF THE NUMBER OF HYBRIDOMA BEARING PLATES WHICH WOULD BE COUNTED AS "FAILURES" BY INCREASING THE MINIMUM REQUIRED DAYS IN CULTURE.

MINIMUM DAYS	TOTAL PLATES SURVIVING	HYBRID BEARING PLATES NOT SURVIVING THIS TIME IN CULTURE.
24+	231	0
30-39	174	0
40-49	122	3
50-59	93	6
60-69	70	10
70+	43	15

"LY" = Lymphocytes.

4.3.2. Hybridomas.

In this study, 25 hybridomas were obtained from 231 successful plates(5544 wells). This gives a hybridoma rate of 0.108 hybridomas per 24 well plate, or 0.004 hybrids/well. This may be a falsely low hybrid rate. Table 5 shows the way in which an increased "minimum days required in culture"would apparently improve the hybrid rate by decreasing the number of plates included in the study. This will be discussed in section 5.2.1..These hybridomas will be discussed further in following paragraphs.

4.4. Fusion Experiments Using PWM Stimulation

4.4.1. Preliminary Experiments.

Two experiments were initially performed to evaluate the "macro" technique for PWM stimulation and to assess the value of cell counting to measure the effects of PWM stimulation.TNLE 184 and TNLE 178 were stimulated with PWM as described.After nine days in culture the cells were examined under phase contrast microscopy , and counts of both the total and large blast cells made by two independent observers. The mean counts obtained are recorded on table 6. As can be seen no definite pattern can be discerned from these counts and in particular no effect of PWM can be demonstrated.

The usefulness of the various means described for estimating the effect of PWM were then assessed over a shorter time period (3 days) using TNLE 205 which was stimulated as described in section 3.3.. The results of this are shown on table 7. From this experiment it appeared that the technique of tritiated thymidine

uptake was the preferred method by which to investigate the effects of PWM stimulation of lymphocytes since this assay most clearly demonstrated the effects of PWM. Therefore a series of five experiments were performed using the "micro" system and the effects of PWM assessed by H3 thymidine uptake. Cells used in these experiments came from T.N.L.E. 271-5, and PWM was added as described previously. The results of these experiments are shown in the graphs in fig. 5-9 . From these graphs it was observed that the level of H^3 uptake diminished from day 2 to day 10 in culture. Since no fresh medium was added to these cells during incubation, it was thought that this may be the result of spent medium in the wells. Three further experiments were performed using T.N.L.E. 282,286 and 288 to assess whether regular feeding of the cultures made a difference. The cells were set up in identical manner but in duplicate plates. One plate was fed by removing 100ul of medium and replacing it with fresh medium, the other plate being fed in like manner but with medium containing PWM. This was to determine a) whether the replacement of spent medium would increase the level of H^3 Thymidine uptake in the longer term cultures and b) whether the longer cultures required the continuous presence of PWM or whether,once stimulated ,the same level of H3 thymidine uptake would be obtained by feeding with medium without PWM. The results of these experiments are shown in figure 10-15. As can be seen TNLE 282 showed a different result from the others in that the concentration of PWM required to produce stimulation in this node is higher than for other nodes and the level of stimulation produced is much lower. It is thought that this represents a degree of anergy in this node.

TABLE 6.

POKEWEED MITOGEN-PRELIMINARY EXPERIMENTS.

CELL COUNTS AS A MEASURE OF THE EFFECT OF POKEWEED MITOGEN.

Lymph Node	Pokeweed Mitogen Concentration (ug/ml)	Cell Counts (x105/ml) Total	Large Cells	Blast (%)
TNLE 178	control	14.1	1.6	11.3%
	1	6.9	2.2	31.8%
	4	6.9	1.6	23.2%
	16	3.1	0.4	12.9%
TNLE 184	control	24	0	0 %
	1	54	5.9	10.9%
	4	56	2.2	3.9%
	16	60	7.6	12.7%

Counts Taken After 9 Days in Culture.

Counts of total cells and large cells morphologically resembling blast cells were made by two independent observers. Three counts were made of each suspension and the mean cell count is recorded. No differences occurred between the two observers.

TABLE.7.

POKEWEED MITOGEN. PRELIMINARY EXPERIMENTS.

Comparison of Four Methods For Measuring the Effects of Pokeweed Mitogen Using TNLE 205 After 3 Days in Culture.

Dose of PWM.	Cell Counts			Rosette Formation
(ug/ml)	Total	Large(Blast)	%Blast	%Rosettes
Control	80	6		15
1	64	13		15
4	85	5		16
16	105	9		22

Dose of PWM	Immunoperoxidase (%)		H^3 Thymidine Uptake
(ug/ml)	anti IgG	anti IgM	(Counts-mean +/-SD)
Control	11.6	21	7071
1	10.0	25	45,918
4	9.8	23.6	46,193
16	5.5	0	47,174

The pattern obtained from TNLE 286 and 288 is very comparable to that obtained from the other experiments, irrespective of whether the cells were fed with normal medium or medium plus PWM. The overall conclusion from these studies was that the stimulation of lymphocytes by PWM forms a plateau type of dose-response curve -ie there is a level of PWM concentration above which no further stimulation of lymphocytes occurs. This presumably represents the point at which all sensitive lymphocytes have been stimulated and this appears to occur at between 2 and 4 ug/ml of PWM. It is also interesting that ,of the times tested,the maximal stimulation occurred at 2 days and decreased thereafter in spite of feeding the cultures. More frequent sampling would have been necessary to confirm that two days was the optimum time period.This pattern is thought to represent the fact that blast transformation occurs early following PWM stimulation and following this the cells may go into a resting,small cell stage.

4.4.2. Fusion Experiments with PWM.

The effects of prestimulating lymphocytes with PWM in the efficiency of hybrid production were assessed as follows. Firstly lymphocytes were stimulated with PWM in two regimes- the one at 2ug/ml of PWM for 48 hours and the other at 16ug/ml of PWM for 5 days. Lymphocytes stimulated at 2 ug/ml for 48 hours were fed with HAT medium, whilst two series of experiments were performed using lymphocytes stimulated at 16 ug/ml for 5 days, the one series being fed with HAT and the other with HAzT medium . In all these series the fusion experiments were performed in matched pairs. At the time of obtaining the node,one half of the node was fused immediately, whilst the other half was first stimulated with PWM. In all other respects the plates were treated in an identical

manner in order that an accurate assessment could be made of the effects of PWM. The results of these experiments are shown in table 8,9 and 10. From table 8 it can be seen that no beneficial effect was gained through stimulation of lymphocytes with PWM at 2 ug/ml for 48 hours and feeding the fusion cultures with HAT medium. One problem with this series is the high number of technical failures due in part to these fusions occurring early in the series . Thus whilst there were 17 successful plates in the "HAT only " column and 18 in the"HAT + PWM 2ug/ml" column, only 10 matched pairs of plates survived long enough in culture to allow direct comparison. Only one of the two hybrids occurred within these matched pairs. Thus the comparison of the sum totals in each column may be slightly inaccurate in that direct comparison of matched pairs was not made throughout the series.. This problem occurred, although to a lesser extent, in all of the other groups of fusion experiments.

Table 9 shows the comparison of "HAT only" and "HAT + PWM 16ug/mlfor 5 days". In this series only two plates were failures, resulting in 16 matched pairs.As can be seen the addition of PWM in this series conferred no increase in the rate of hybrid production.

Table 10 shows the comparison between the "HAzT only" and the "HAzT + PWM16 ug/mlfor 5 days" series of plates. From these results the number of hybrids was approximately equal in the two columns but is considerably higher than the equivlent series using HAT. In the series using HAzT only two hybrids (from plate FE 59/3) grew in a plate where the matched plate (FE59/1) was a technical failure.

TABLE 8.

POKEWEED MITOGEN - FUSION EXPERIMENTS.

1) Effect of Preincubating Lymphocytes with PWM at 2 ug/ml for 48 Hours Prior to Fusion in H.A.T. Medium.

Control			PWM Stimulated Cells.		
Plate	Infective Agent (Time)	Hybrid (Time)	Plate	Infective Agent (Time)	Hybrid (Time)
21/1	B 9	) same (	22/1	B 5	
21/2	Y 10	)fusion(	22/2	Y 7	
26/3	M 69		26/1	Y 59	
26/4	Y 59		26/2	M 69	
27/3	Y 20		27/1	Y 20	
27/4	Y 19		27/2	Y 27	
28/3	B 28		28/1	B 28	
28/4	B 28		28/2	B 28	
29/3	Y 12		29/1	Y 12	
29/4	Y 39		29/2	Y 12	
30/3	B 17		30/1	B 17	
31/4	M 47		31/2	M 25	
32/1	Y 52	H 36	32/3	M 48	
35/4	M 28		35/2	M 22	
36/2	M 35		36/4	M 27	

TABLE 8 (Continued

37/1	M	26	37/2	Y	41
41/2	M	21	41/3	M	28
41/2A	M	21	41/3A	M	28
			41/4	M	28
			41/4A	M	28
42/2	M	34	42/4	M	33
43/1	M	38	43/3	M	23
44/1	B	19	44/3	M	25
44/2	M	21	44/4	M	23
45/4	M	68	45/2	M	65
47/2	M	45	47/4	)Unknown	
47/2A	unknown		47/4A	)	
48/2	M	90	48/4	Y	5
50/2	B	7	50/4	B	7
55/2	M	19	55/4	M	19
16 Successful Plates			16 Successful Plates		

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13 "Failures"	13 "Failures"
1 Unknown	3 Unknown
2 Hybrids	0 Hybrids

(10 successful matched pairs of plates)

(B=Bacteria,M=Mould,Y=Yeast, Time in Days).

TABLE 9.

POKEWEED MITOGEN- FUSION EXPERIMENTS.

The Effect of Prestimulating Lymphocytes with PWM at 16ug/ml for 5 Days Prior to Fusion in HAT Medium.

Control			PWM 16ug/ml for 5 Days		
Plate	Infecting Agent (Time)	Hybrid (Time)	Plate	Infecting Agent (Time)	Hybrid (Time)
69/1	M 108	Hybrid 60	69/3	M 70	
73/1	M 27		73/3	M 94	
73/2	Y 54		73/4	M 91	
79/1	M 112		79/7	M 47	
79/2	M 55		79/8	M 96	
80/1	M 10		80/7	M 59	
80/2	M 38		80/8	M 38	
83/1	M 33		83/7	M 40	
83/2	M 19		83/8	M 26	
84/1	M 37		84/7	M 12	
84/2	M 97		84/8	M 26	
85/1	M 64		85/5	M 35	
85/2	M 39		85/6	M 31	

TABLE 9 {CONTINUED}.

89/1	M	107	89/3	Y	96
89/2	M	104	89/4	M	24
93/1	Y	19	93/3	M	103
93/2	M	119	93/4	M	103
94/1	M	73	94/3	M	84
94/2	M	66	94/4	M	77

16	Successful Plates	18	Successful Plates
3	"Failures"	1	"Failures"
1	Hybrid	0	Hybrid

(15 Successful pairs of Matched Plates)

(B=Bacteria,M=Mould,Y=Yeast, Time in Days)

(Time is in Days)

TABLE 10.

POKEWEED MITOGEN - FUSION EXPERIMENTS.

The Effect of Prestimulation of Lymphocytes with PWM at 16ug/ml for Five Days Prior to Fusion in HAzT Medium.

Control				PWM 16ug/ml			
Plate	Infecting Agent (Time)		Hybrid (Time)	Plate	Infecting Agent (Time)		Hybrid (Time)
49/1	B	10		49/3	M	37	
49/2	B	10		49/4	M	37	
51/1	M	60		51/3	M	60	
51/2	M	25		51/4	Y	45	Hybrid 28
52/1	M	21		52/3	M	39	
52/2	M	48		52/4	M	48	
53/1	M	51		53/3	M	31	Hybrid 31
53/2	M	31		53/4	M	49	Hybrid 49
54/1	M	12		54/3	M	58	
54/2	M	31		54/4	M	54	Hybrid 41
56/1	M	42		56/3	M	44	
56/2	M	24		56/4	M	42	

TABLE 10 {CONTINUED}

59/1	Y	19		59/3	M	30	Hybrids x2 Day 24
59/2	Y	19		59/4	M	42	
60/1	M	38		60/2	M	38	
60/2	M	14		60/4	M	38	
63/1	M	24		63/3	M	28	
63/2	M	24		63/4	M	24	
64/1	M	21		64/3	M	17	
64/2	M	97		64/4	M	25	
67/1	M	52		67/3	M	106	
67/2	M	66	Hybrid 46	67/4	M	77	
69/2	M	136		69/4	M	96	
71/1	Y	38	Hybridx2 35	71/3	Y	47	
71/2	M	74	Hybridx2 38	71/4	M	96	
74/1	Y	36		74/3	M	130	
74/2	Y	60	Hybridx2 36	74/4	M	60	

19	Successful Plates	26	Successful Plates
8	Failures	0	Failures
7	Hybridomas	6	Hybridomas

19 Successful Matched Pairs of Plates

(B=Bacteria, Y=Yeast, M=Mould, Time in Days.)

(Time is in Days)

4.5. Feeder Cells.

4.5.1. Monocyte Feeder Cells. Preliminary Experiments.

Monocytes were separated from the lymphocytes by their ability to adhere to plastic surfaces. This property of monocytes has been described by Kumagai et al.,(1979),Treves et al.,(1980), Ackerman et al.,1978, Territo et al.,(1977). These studies have shown that monocytes adhere to plastic surfaces if incubated for 1-2hours at 37°C . If however they are incubated for a prolonged time (greater than 12 hours) this property is lost.

Preliminary experiments were performed to test this phenomenon. Cell counts of the number of monocytes (large cells morphologically resembling monocytes) and total cells were made of the lymphocyte /monocyte suspension. This suspension was then distributed over the wells of a 24 well plate (Nunc) at a concentration of $5x10^4$ cells/well,and incubated at 37°C for varying times between 30 min. and $2\frac{1}{4}$ hours. The cells were then removed from the wells by gentle suction followed by gentle irrigation of the wells with TCM-A/2%FCS. Cells removed by suction and irrigation were then centrifuged, resuspended and counted, and the cells left adherent to the wells calculated by subtraction from the original counts. Table 11 shows the cell counts obtained by using this technique and shows that the incubation time appeared to be of little importance except that the two hour culture showed fewer cells adherent. The plates were then examined microscopically after staining the cells with Giemsa stain and this showed a high density of adherent monocyte cells in the first three incubation times but a reduced density in the 2 hour plate. From these results, one hour was adopted as the standard incubation time for monocyte adherence.

Further experiments were conducted to determine how many monocytes needed to be added to leave a minimum of $5x10^4$ cells adherent to each well. Table 12 summarises the results of these experiments. On average approximately 85% of monocytes were found to be adherent after one hour of incubation. It was decided therefore that, to leave a minimum of $5x10^4$ cells/well, 10^5monocytes in suspension would be incubated in each well for one hour prior to fusion.

On the morning of a fusion experiment a blood sample was prepared as described, and the mononuclear cell suspension distributed over the wells of the 24 well culture plate at a concentration of $5x10^4$ cells/well.The plate was incubated for one hour at 37^oC in the CO_2 incubator and then the cell suspension was aspirated and the wells gently irrigated with TCM-A/2%FCS. Thereafter the plate was used as previously described for the fusion experiment .

4.5.2. Monocytes - Fusion Experiments.

The effect of adding monocytes to the fusion experiments is illustrated in table 13 . In addition to the simple addition of monocytes to these cultures the effects of adding monocytes to fusion experiments,involving lymphocytes already pre-stimulated with PWM at 2 ug/ml for 48 hours, was assessed and these results are shown in table 14. The addition of monocytes did not improve the efficiency of hybrid production. This series contained a relatively high number of "failures" -some of these experiments were performed in the early part of the study. Neither did the combination of PWM and monocytes confer any benefit on the rate of hybrid production .

TABLE 11.

MONOCYTE FEEDER CELLS - PRELIMINARY EXPERIMENTS.
Effect of Varying Incubation Time on Monocyte Adherence.

Incubation Time (Minutes)	Monocyte Counts (a) Initial Count	Monocytes Retrieved	Adherent (b) Monocytes(%)
30	6.4	0.3	6.1 (95.3%)
60	6.4	0	6.4 (100%)
90	6.4	0	6.4 (100%)
120	6.4	1.2	5.2 (81.3%)

(Monocyte Counts all $x10^5$/ml)

(a) Monocyte counts are the mean of 3 counts of each cell suspension.

(b) Adherent monocytes are calculated by deduction. Preliminary experiments using Giemsa stain confirmed the presence of many adherent monocytes.

TABLE 12.

Effect of Varying Monocyte Input on Monocyte Adherence.

Monocyte Counts			
Initial Count	Monocytes Retrieved	Adherent Monocytes(%)	(by deduction)
3.7	1	2.7	(72.9%)
7.4	1	6.4	(86.5%)
15	2	13	(86.6%)
20	2	18	(90.0%)
29	4	25	(86.2%)
37	5	32	(86.5%)

(All cell counts x104/ml.)

TABLE 13.

MONOCYTE FEEDER CELLS - FUSION EXPERIMENTS.

The Effect of the Addition of Monocyte Feeder Cells on the Rate of Hybridoma Production Using HAT Medium.

Control			Monocyte Feeder Cells.		
Plate	Infecting Agent (Time)	Hybrid. (Time)	Plate	Infecting Agent (Time)	Hybrid (Time)
31/4	M 48		31/3	M 48	
32/1	Y 55	Hybrid 35	32/2	M 35	
34/4	Y 51		34/3	M 38	
35/4	M 28		35/3	M 20	
36/2	Y 35		36/1	M 21	
40/2	B 3		40/1	B 3	
40/2A	B 3		40/1A	B 3	
40/4	Y 41	Hybrid 32	40/3	Unknown	
40/4A	Y 61	Hybrid 33	40/3A	Unknown	
42/2	M 34		42/1	Y 68	
45/2	Y 60		45/1	B 12	
47/2	M 54		47/1	M 54	
47/2A	Unknown		47/1A	M 61	
48/2	Y 89		48/1	Y 82	

TABLE 13 {CONTINUED}

50/2	B	7	50/1	B	7
55/2	M	19	55/1	M	11
79/1	M	117	79/5	M	131
79/2	M	55	79/6	M	96
80/1	M	110	80/5	Y	73
80/2	M	38	80/6	M	76
83/1	M	33	83/6	M	33
84/1	M	37	84/5	Y	121
84/2	M	97	84/6	M	63

18	Successful Plates	14	Successful Plates
3	"Failures"	6	"Failures"
1	Unknown	2	Unknown
3	Hybridomas	0	Hybridomas

13 Successful Pairs of Matched Plates

(B=Bacteria, Y=Yeast, M=Mould, Time in Days)

TABLE 14.

COMPARISON OF THE EFFECT OF THE COMBINATION OF MONOCYTE FEEDER CELLS AND PRESTIMULATION OF LYMPHOCYTES WITH PWM AT 2ug/ml ,USING HAT MEDIUM.

Control			"Monocyte + PWM" Group.		
Plate	Infective Agent (Time)	Hybrid (Time)	Plate	Infective Agent (Time)	Hybrid. (Time)
31/4	M 47		31/1	M 32	
32/1	Y 52	H 36	32/4	M 42	
34/4	Y 50		34/1	M 35	
35/4	M 27		35/1	M 27	
36/2	Y 35		36/3	M 36	
37/1	Y 31		37/3	Y 42	
42/2	M 34		42/3	Y 68	
45/2	Y 37		45/3	M 40	
47/2	M 54		47/3	Y 89	
47/2A	M 54		47/3A	M 54	
48/2	Y 89		48/3	B 4	
50/2	B 7		50/3	B 7	
55/2	M 18		55/3	M 18	
11 Successful Plates			10 Successful Plates		
2 Failures			3 Failures		
1 Hybrid			0 Hybrids.		

10 Successful Pairs of Matched Plates.

(B=Bacteria, Y=Yeast, M=Mould, Time in Days.)

4.5.3. Thymocytes.

The addition of thymus feeder cells was seen to slightly improve the rate of hybrid production as shown in table 15. In this series only 3 plates were failures and 13 matched pairs were successful. The effect of thymocyte feeder cells and PWM stimulation together was not assessed in the course of this study.

TABLE 15.

THYMOCYTE FEEDER CELLS - FUSION EXPERIMENTS.

The Effect of Adding Thymocyte Feeder Cells on the Rate of Hybridoma Production Using HAT Medium.

Control			Thymocyte Feeder Cells		
Plate	Infecting Agent (Time)	Hybrid (Time)	Plate	Infecting Agent (Time)	Hybrid (Time)
38/2	M	30	38/1	M	30
38/4	Y	26	38/3	M	28
39/2	M	27	39/1	M	27
39/4	M	58	39/3	B	32
41/2	M	21	41/1	M	29
41/2A	M	21	41/1A	M	29
65/2	M	42	65/1	M	130
66/3	M	31	66/1	Y	31
66/4	M	52	66/2	Y	31
72/2	M	46	72/1	M	25
75/3	M	17	75/1	M	17
75/4	M	107	75/2	M	28

TABLE 15 (cont.)

76/3	M	112	76/1	M	69 Hybridx2
					53
76/4	M	112	76/2	M	58
85/1	M	64	85/3	M	95
85/2	M	39	85/4	M	60

13 Successful Plates	15 Successful Plates
3 "Failures"	1 "Failures"
0 Hybridomas	2 Hybridomas

13 Successful Pairs of Matched Plates.

(B=Bacteria, Y=Yeast, M=Mould, Time in Days).

4.6. Selective medium

In this series of experiments matched pairs of fusion plates were fed with either HAT or HAzT medium. The effect of these selective media is shown on table 16. Whilst 2 hybrids were obtained from the HAT group and none from the HAzT group, the number of plates involved was small and ,as will be discussed the overall analysis of the hybridomas obtained in this study suggest that HAzT is a more efficient hybrid producer than HAT

4.7. Cell Numbers.

The effect of varying the lymphocyte/myeloma ratio is shown in table 17. HAT medium was used in these experiments. From this series no particular ratio is seen to be more beneficial than any other, although the number of plates in this series is smaller than in the others.

TABLE 16.

SELECTIVE MEDIA - FUSION EXPERIMENTS

Comparison of the Effects of Using Either HAT or HAzT as Selective Medium on the Rate of Hybridoma Production.

HAT			HAzT		
Plate	Infecting Agent (Time)	Hybrid (Time)	Plate	Infecting Agent (Time)	Hybrid (Time)
69/1	M 108	Hybrid 60	69/2	M 136	
77/1	M 58	Hybrid 36	77/3	Y 35	
77/2	Y 36		77/4	Y 35	
78/1	M 31		78/3	M 119	
78/2	M 17		78/4	M 119	
79/1	M 117		79/3	M 40	
79/2	M 55		79/4	M 56	
80/1	M 110		80/3	M 38	
80/2	M 38		80/4	M 76	
81/1	M 41		81/3	M 27	
81/2	M 34		81/4	Y 41	
82/1	M 33		82/3	M 26	
83/1	M 33		83/3	M 26	
83/2	M 19		83/4	M 33	
84/1	M 37		84/3	M 37	
84/2	M 97		84/4	M 107	

TABLE 16 {CONTINUED}

14	Successful Plates	16	Sucessful Plates
2	"Failures"	0	"Failures"
2	Hybridomas	0	Hybridomas

14 Successful Pairs of Matched Plates.

(B=Bacteria, Y=Yeast, M=Mould, Time in Days, HAT= Hypoxanthine, Aminopterin and Thymidine, HAzT= Hypoxanthine, Azaserine, and Thymidine.)

TABLE 17

THE EFFECT OF VARYING CELL INPUT ON THE RATE OF HYBRID PRODUCTION

CELL RATIO {LYMPHOCYTE/MYELOMA}					
10:1		5:1		1:1	
PLATE	RESULT	PLATE	RESULT	PLATE	RESULT
87/1	M32	87/2	M12	87/3	M20
88/1	M12	88/2	M20	88/3	Y66
91/1	M66	91/2	M32	91/3	Y44
95/1	M56 H41	95/2	M65	95/3	M65
96/1	M58	96/2	M62	96/3	M62 H45
97/1	M39	97/2	M56	97/3	M53
98/1	Y50	98/2	Y46 H42	98/3	M144 H69
99/1	M44	99/2	M44	99/3	M40
100/1	M24	100/2	M27	100/3	M53
101/1	M61	101/2	M41	101/3	M48

TOTALS			
PLATES	10	10	10
FAILURES	1	2	1
HYBRIDS	1	1	2

(M=Mould, Y=Yeast, H=Hybridoma, Number represents time in days after fusion at which these events occurred.)

4.8. Hybridomas.

Table 18 summarises the hybrids obtained, the time after fusion at which they appeared, the culture conditions under which they were grown, and their ultimate fate.

Hybrids appeared at widely differing times after cell fusion, the mean being 42.2 days, the range 24-69 days. No matter how long after fusion the hybrids appeared there seemed to be no noticeable difference in their growth rates in culture-ie a hybrid which did not appear until late after fusion did not appear thereafter to be a slow growing hybrid.

Table 19 relates the percentage of hybrids obtained compared to the total number of plates for each set of culture conditions. From these figures it is seen that plates fed with HAzT produced a higher percentage of hybrids than plates fed with HAT, whatever other culture conditions were varied.This was different from the results obtained when the two were compared directly. This may suggest that HAzT is a more efficient medium for hybrid production that HAT.

One disappointing aspect of these hybrids was the number which failed to survive in long term culture. Only 9 hybrids grew well in culture and could be characterised. Öf the remainder, 2 were lost because of bacterial contamination during medium changing. These hybrids had been growing well until that point and were being subcultured when the contamination occurred. The remainding 14 hybrids were lost at the stage of transferring the cells from the 24 well plates into bulk culture.

TABLE 18.

SUMMARY OF HYBRIDOMAS OBTAINED.

Hybridoma	Time After Fusion (Days)	Culture Conditions Sel /Cell /PWM /Feeder Med. Ratio Cells	Progress
32/1/B4	36	HAT / 10:1/ - / -	S
40/4/A4	32	HAT / 10:1/ - / -	D
40/4A/D3	33	HAT / 10:1/ - / -	D
51/4/A6	28	HAzT/ 10:1/ 16 / -	D
53/3/A2	31	HAzT/ 10:1/ 16 / -	S
53/4 B4	49	HAzT/ 10:1/ 16 / -	S
54/4/D4	41	HAzT/ 10:1/ 16 / -	S
59/3/A3	24	HAzT/ 10:1/ 16 / -	D
59/3/B1	29	HAzT/ 10:1/ 16 / -	D
67/2/A3	46	HAzT/ 10:1/ - / -	D
69/1/A3	60	HAT / 10:1/ - / -	D
71/1/A6	35	HAzT/ 10:1/ - / -	D
71/1/B4	35	HAzT/ 10:1/ - / -	D
71/2/A1	38	HAzT/ 10:1/ - / -	S
71/2/A2	38	HAzT/ 10:1/ - / -	S
71/2/B4	66	HAzT/ 10:1/ - / -	D
73/3/B1	65	HAT / 10:1/ 16 / -	D
74/2/D2	36	HAzT/ 10:1/ - / -	S
74/2/D4	36	HAzT/ 10:1/ - / -	S

TABLE 18 (cont.)

76/1 D1	53	HAT / 10:1/ - / T	D
77/1/B4	36	HAT / 10:1/ - / -	D
95/1/C3	41	HAT / 10:1/ - / -	D
96/3/D5	45	HAT / 1:1/ - / -	D
98/3/D6	69	HAT / 1:1/ - / -	D

Cell Ratios are Lymphocyte/Myeloma.

"T"= Thymocytes

"S"= Survived in Culture

"D"= Died in Culture

"16"= Prestimulation of Lymphocytes in Pokeweed Mitogen at 16ug/ml for 5 Days Prior to Fusion

TABLE 19.

Summary of the Success Rate of the Different Options Employed for Cell Fusion and Culture.

Fusion / Culture Conditions	Hybridomas	Successful Plates	Hybridomas/ Platesx100
HAT	6	72	8.3%
HAzT	8	38	21.0%
HAT/PWM16	1	20	5.0%
HAzT/PWM16	6	26	23.1%
HAT/Thymocytes	2	15	13.3%
HAT/1:1Cell Ratio	2	10	20.0%

"PWM16" = Prestimulation of Lymphocytes with Pokeweed Mitogen at 16ug/ml for 5 days

All Fusions with a 10:1 Lymphocyte Myeloma Ratio Unless Otherwise Stated.

Table 20 shows the relationship of the hybrids to the date on which the corresponding fusion experiment was performed . Whilst fusion experiments were performed regularly throughout the course of this study,it appeared that there were clusters of fusion experiments performed within relatively short time intervals which produced many hybridomas. The reason for this is unknown and will be discussed later.

TABLE 20.

Relationship of Appearence of Hybridomas to the Date on Which the Fusion Experiment was Performed.

Hybridoma	Date of Fusion (Day,Month,Year)
32/1/B4	24/11/82
40/4/A4	27/01/83
40/4A/D3	27/01/83
51/4/A6	07/03/83
53/3/A2	22/03/83
53/4/B4	22/03/83
54/4/D4	22/03/83
59/3/A3	19/04/83
59/3/B1	19/04/83
67/2/B3	03/06/83
69/1/A3	10/06/83
71/1/A6	10/06/83
71/1/B4	10/06/83
71/2/A1	10/06/83
71/2/A2	10/06/83
71/2/B4	10/06/83
73/3/B1	20/06/83
74/3/D2	10/06/83
74/2/D4	10/06/83
76/1/C2	18/06/83
76/1/D1	18/06/83
77/1/B4	22/06/83
95/1/C3	07/09/83
96/3/D5	09/09/83
98/3/D6	22/09/83

4.9. Characterisation of Hybrids and Their Supernatants.

As was mentioned previously fusions FE1-19 were not included in this study, but hybrids from these fusions were characterised in the course of this study and these results are included in the data presented. The results of these experiments are now detailed.

4.9.1. Chromosome Count.

The chromosome count of each hybrid is shown in table 21. Each of the counts is the mean of at least 3 different cell nuclei,and were confirmed by an independent observer.In no case did the results vary by more than 5 from the mean. In each case well spread out preparations of individual nuclei were selected for counting to allow as accurate a count as possible. All but one of the hybrids were found to have a chromosome count of less than 92, suggesting that some chromosomes were lost at the time of cell fusion.

Most of the hybrids had their chromosome counts repeated after several weeks in culture and no changes in the chromosome counts were noted, suggesting that the hybrids remained stable in culture over this period in time. A typical chromosome preparation is shown in plate 1.

4.9.2. Cell Surface Markers.

The cell surface markers were performed to determine whether the hybrids arose from fusion of a T or B cell with the HmY2 cell.The results are shown on table 22.In this table also are shown the

TABLE 21

Chromosome Counts of Hybridomas.

Hybridoma	Chromosome Count
1/2/B2	86
1/4/B6	87
1/4/C2	89
4/1/A4	90
4/1/B2	85
4/1/C6	88
4/2/B2	86
4/2/C2	84
5/1/D5	86
6/1/A2	91
6/1/A3	92
6/4/A3	80
32/1/B4	87
53/3/A2	78
53/4/B4	85
54/4/D4	89
71/2/A1	87
71/2/A2	83
74/4/D4	74

Mean Chromosome Count 85.63. Range 74-92.

PLATE 1

Chromosome preparation of hybridoma F.E. 1/2/B2

(Magnification x160)

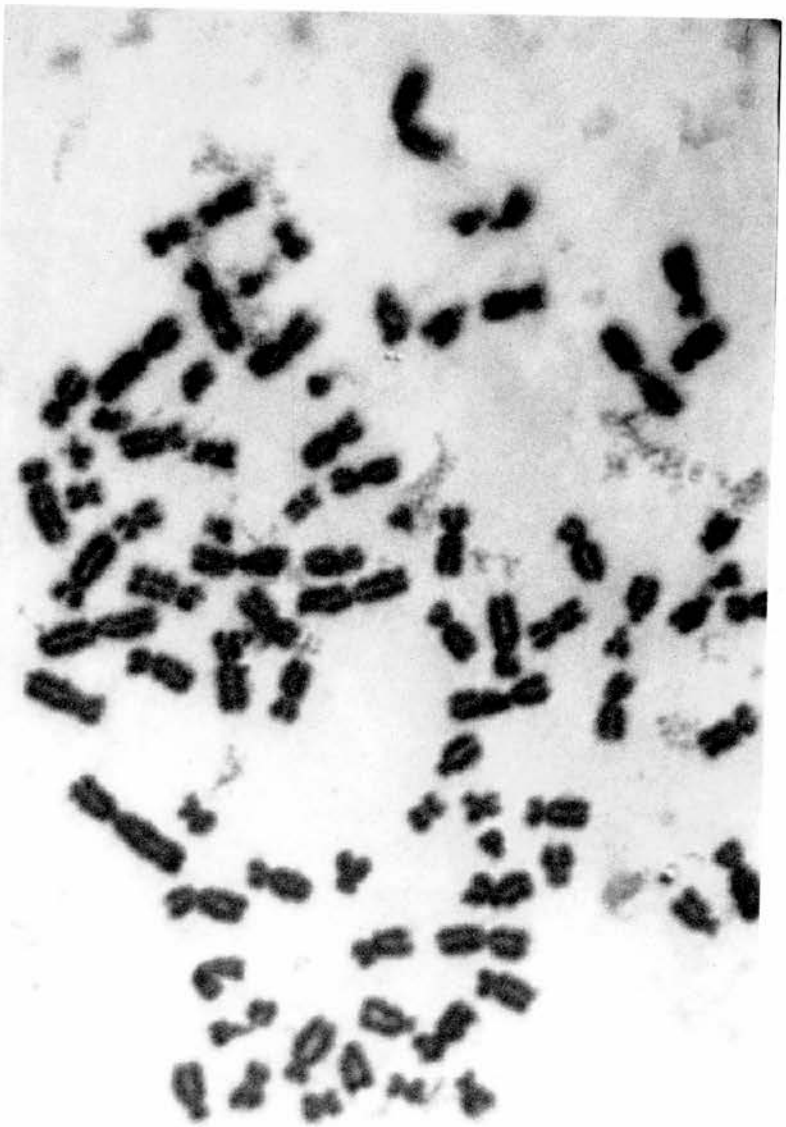


TABLE 22.

Cell Surface Markers of Hybridomas as Defined Rosetting Reaction Described in Section 3.6.2.

Hybridoma	Rosettes		
	T-cell	Sheep anti-human Fab	Normal Sheep Immunoglobulin
32/1/B4	4	17	1
53/3/A2	7	56	6
53/4/B4	2	63	7
54/4/D4	0	31	3
71/2/A1	4	60	4
71/2/A2	1	22	2
71/2/B4	0	25	10
74/2/D2	1	41	3
74/2/D4	1	37	2
HMy2	1	75	7
TNLE 192	56	27	0
TNLE 217	48	20	0
TNLE 238	60	25	0
TNLE 240	53	26	0

NUMBERS ARE THE PERCENTAGE OF ROSETTING CELLS

results of rosettes formed by SRBC's coated with normal sheep immunoglobulin. This served as a negative control to ensure that there were no non-specific rosettes formed. Also included in this table are the results of rosettes formed from lymphocyte suspensions from four axillary nodes (TNLE 192,217,238,240.) to show the normal distribution of T and B cells within these nodes. It is concluded from this table that the hybrids all arose from B-lymphocytes and there is no evidence of T-cell hybrids having been formed.

4.10. Screening of Supernatants.

4.10.1. Measurement of Immunoglobulin Production.

Estimation of immunoglobulin production was made by an agglutination reaction using SRBC's coated with the appropriate antibody. When each batch of SRBC's was coupled control agglutination reactions were performed to ensure that agglutination was not occurring in a non-specific way. Each batch of coupled SRBC's was therefore reacted with normal human serum as a positive control and normal sheep serum as a negative control. Any batch of coupled SRBC's failing to react appropriately with either of these controls was discarded.

The results of these agglutination reactions are shown on table 23. From this it can be seen that most hybrids as well as HMy2 secrete IgG, although the sub-class of IgG was not determined using this assay. A few hybrids were found however to secrete

both IgG and IgM. Whilst it was not possible to say on the basis of this assay that the hybrids secreted a different IgG from the HMy2 cell line,the secretion of IgM by some hybrids is proof that these hybrids secrete new (and different) immunoglobulin chains from the HMy2.

This reaction was also used to determine the concentration of immunoglobulin secreted by the hybrids. Control human serum was therefore obtained and was found to have an IgG concentration of 13.7g/l and an IgM concentration of 1.0g/l (courtesy of the Dept of Clinical Chemistry, Royal Infirmary of Edinburgh). By using this serum the hybrid supernatants were found to contain the concentrations of immunoglobulin shown on table 23.

TABLE 23.

Type and Concentration of Immunoglobulin Secreted by Hybridomas, by the Agglutination Reaction.

Hybridoma	Immunoglobulin Secreted	Immunoglobulin Concentration
1/2/B2	IgG	
1/4/B6	IgG + IgM	
1/4/C2	IgG + IgM	
4/1/A4	IgG	
4/1/B2	IgG + IgM	
4/1/C6	IgG	
4/2/C2	IgG + IgM	
5/2/D5	IgG	
6/1/A2	IgG	
6/1/A3	IgG	
6/4/A3	IgG + IgM	
32/1/B4	IgG	0.2ug/ml
	IgM	1.2ug/ml
53/3/A2	IgG	0.2ug/ml
53/4/B4	IgG	0.01ug/ml
54/4/D4	IgG	0.2ug/ml
71/2/A1	IgG	0.82ug/ml
71/2/A2	IgG	0.82ug/ml
74/2/D2	IgG	0.2ug/ml
74/2/D4	IgG	
76/1/D1	IgG	
76/1/C2	IgG	
HMy2	IgG	0.2ug/ml

4.10.2. The Radioimmunoassay.

In each of the assays performed, control wells were included to ensure that any binding detected in the assay represented a specific binding of the McAb to the target antigen.

As a negative control,both normal human serum and HMy2 supernatant were used in place of the hybridoma supernatant, since neither of these should react specifically with breast carcinoma cells and would therefore give an indication of the amount of non-specific binding of human immunoglobulin to the wells of the plate. In addition the first antibody stage (either the hybridoma supernatant or HMy2 supernatant) was omitted in three wells of each plate to ensure that no non-specific binding of the other two antibodies was occurring. The mouse anti human kappa and lambda antibodies were also omitted from three wells to demonstrate the degree of nonspecific binding of the iodinated rabbit anti-mouse antibody.

An appropriate positive control was more difficult since a human McAb with specificity for breast cancer cells was not available. Instead NIBBS (human anti-HLA serum, see apendix 1) was used to bind onto the CLA4 cell line (a lymphoblastoid cell line).This combination would provide a human immunoglobulin bound onto a human cell line, and could therefore be used as a positive control.

The radioimmunoassay as described was found to produce very high levels of background counts (of the order of 400 - 2000 counts per well). This occurred in control wells where HMy2 supernatant was used in place of hybrid supernatant, and also in wells in which both the hybridoma supernatant and the mouse anti human kappa (lambda) antibodies were omitted. It was concluded that this high background

was caused by non-secific binding of the radioiodinated rabbit anti-mouse antibody to the plastic of the wells of the plate. Therefore,instead of merely adding 50ul of blocking buffer (see page 90 for details) to each well of the plate, the whole plate was immersed in blocking buffer. This attempt to reduce the high level of background binding was unsuccessful.

Careful attention was paid to the washing of the plates between the addition of each of the antibodies. The amount of radiolabelled antibody added to each well was reduced,but neither of these measures reduced the degree of background binding. A total of 28 assays were performed but in none of these could the level of background be reduced to acceptable levels.

In addition to the high background counts obtained, no difference was observed in the counts obtained from the positive and negative control wells. Whilst any difference between these wells may have been obscured by the high level of background, the experimental system was further investigated. The mouse anti human kappa and lambda chain antibodies were conjugated to SRBC's, to determine whether these conjugated SRBC's would then agglutinate in the presence of control preparations of human kappa and lambda chains.However no agglutination was seen to occur.

This agglutination reaction included appropriate controls to ensure that the coupling reaction had been successful.For these controls mouse anti rabbit immunoglobulin antibody (Dako) was conjugated to SRBC's at the same time as the mouse anti human kappa and lambda antibodies. These control coupled SRBC's were then found to produce an agglutination reaction in

the presence of normal rabbit serum. It was concluded that mouse immunoglobulin satisfactorily coupled to the SRBC's by the methods used.

In view of these several difficulties and the theoretical advantages in favour of the immunoperoxidase technique, no further use was made of the radioimmunoassay in this study.

4.10.3. The Immunoperoxidase Technique.

a) The Controls

Before the immunoperoxidase technique was used to determine the binding of hybridoma supernatants to breast cancer cells numerous control experiments were performed to assess the accuracy of the technique.

A positive control was difficult to devise as no human McAb with specificty for breast cancer cells was available. Two different positive controls, therefore, were used to circumvent this problem. Firstly a human antibody which would bind to a different cell type was used as a model on which to work out the appropriate concentrations of the various antibodies used in the assay. For this purpose NIBBS (human anti HLA serum derived from a multiparous woman (Dept. of Immunology, Cambridge)) was used to bind onto cytocentrifuge preparations of the CLA4 cell line as described in the preceding section. Secondly a rabbit antibody was used which bound onto breast epithelium cells, both normal and malignant. For this purpose rabbit anti-human epithelial membrane antigen antibody was used (Sloane et al 1981). This control was used when testing hybridoma supernatants against breast cancer cells.

Using the NIBBS/CLA4 system a number of control experiments were performed. The first of these aimed to determine whether nonspecific binding of any of the antibodies used in the experiment occurred, giving false positive results. This was done by omitting in sequence first each antibody and then combinations of antibodies from the process, and the results are shown in table 24. As can be seen , with one exception when one antibody was

missed out no stain occurred whereas when all antibodies were present a strong stain of virtually all cells was recorded. With this one exception no non-specific binding of any of the antibodies was seen to occur.

The exception occurred when NIBBS alone was ommitted. It appeared therefore, that this weakly positive stain (Line 3 table 24) was due to binding of the rabbit anti human IgM to the CLA4 cell line. To test whether this represented non specific binding of rabbit immunoglobulin to the cell cytocentrifuge preparation the antibody was replaced with normal rabbit serum. This produced no evidence of staining . It was concluded that this staining represented specific binding of the antibody to the cell line,which might be expected since the CLA4 cell line expresses surface immunoglobulin. This binding of the second stage antibody to human immunoglobulin already present on the slide remained a problem throughout the course of this study and will be discussed in greater detail later. A similiar series of controls were performed replacing the rabbit anti human IgM with rabbit anti human IgG and an identical series of results were obtained.

In each group of immunoperoxidase experiments controls were always included which involved missing out the hybridoma supernatant. This allowed the binding of the second stage antibodies (ie the rabbit anti-human IgG/IgM) to the immunoglobulin already present within the tumour section or cytocentrifuge preparation to be demonstrated and compared with the binding pattern obtained from the hybridoma supernatant.

In addition HMy 2 supernatant was also tested with each group of slides to determine whether the immunoglobulin produced by this cell line produced any non specific binding.

The next series of control experiments investigated the optimal dilution of each antibody to produce a reliable result with the minimal background, since too little of any antibody may decrease the sensitivity of the technique whilst too much may increase the level of background binding. A series of experiments was performed in which the concentration of each antibody was varied keeping all others constant.

A 1/100 solution of the rabbit anti human IgG and IgM antibodies and the PAP antibody complex was found to produce clear staining with minimal background,whilst further dilution diminished the intensity of the staining. These dilutions were therefore adopted as standard. The swine anti rabbit antibody was used in a 1/50 dilution since the assay requires an excess of this antibody. The hybridoma supernatants were normally used undiluted because of the low amount of that immunoglobulin they contained. In some cases the supernatants were concentrated X50 in a Minicon protein concentrator but this did not alter the binding patterns obtained.These controls are summarised in figures 3 and 4.

TABLE 24.

Immunoperoxidase Controls

1) Positive Controls -the Use of HLA Serum and the CLA4 Cell Line to Assess the Effects of Excluding Each of the Antibody Steps .

Antibodies				Result
HLA Serum	Rabbit anti Human IgM	S.A.R.	P.A.P.	
+	+	+	+	Positive
+	-	+	+	Negative
-	+	+	+	Weak Positive
+	+	-	+	Negative
+	+	+	-	Negative
-	-	+	+	Negative
+	-	-	+	Negative
+	+	-	-	Negative
+	-	-	-	Negative
-	+	-	-	Negative
-	-	+	-	Negative
-	-	-	+	Negative

(+) = antibody added

(-) = antibody not added

S.A.R. = Swine Anti Rabbit Antibody

P.A.P. = Peroxidase anti Peroxidase Complex

FIGURE 3.

THE IMMUNOPEROXIDASE REACTION.

THE POSITIVE CONTROL EXPERIMENTS.

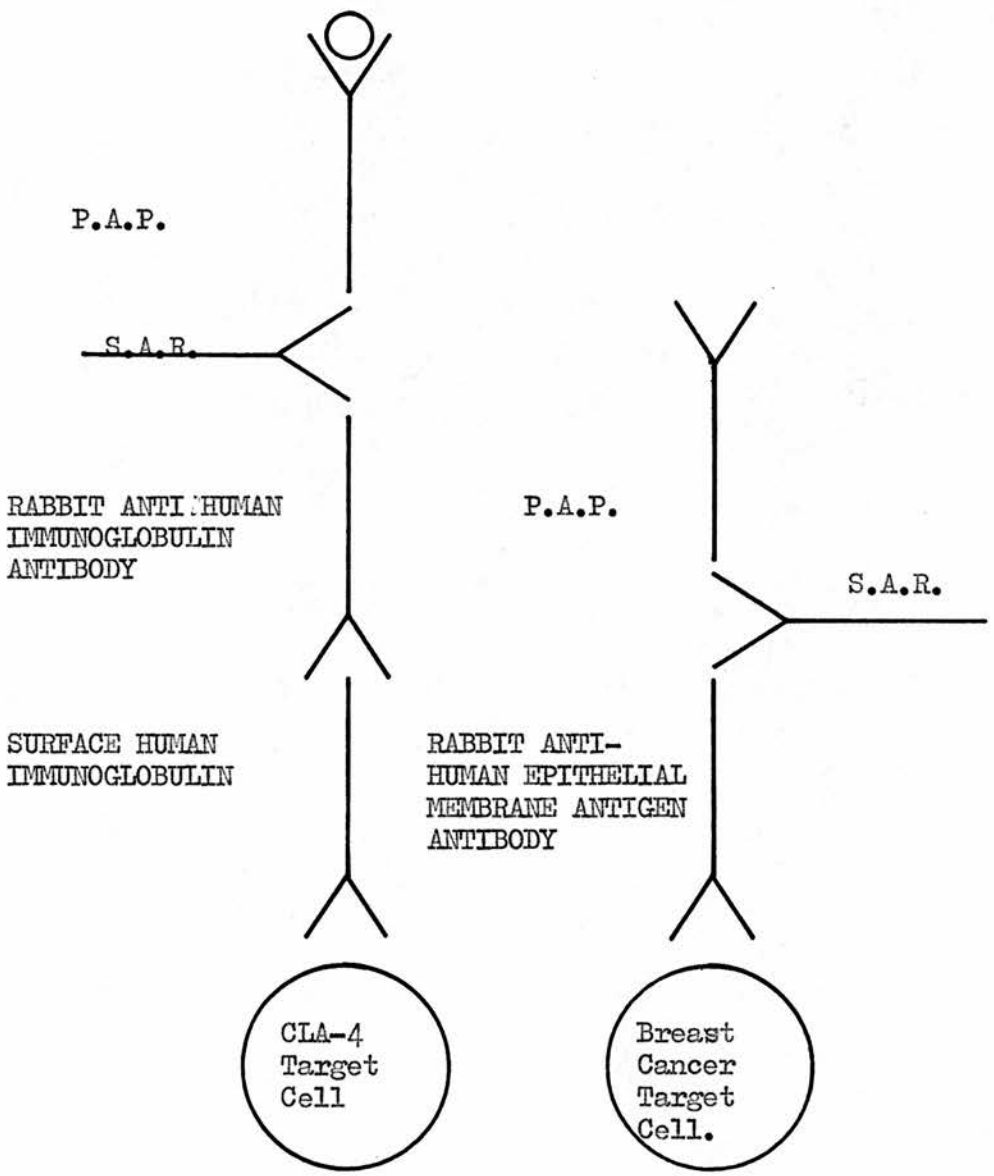
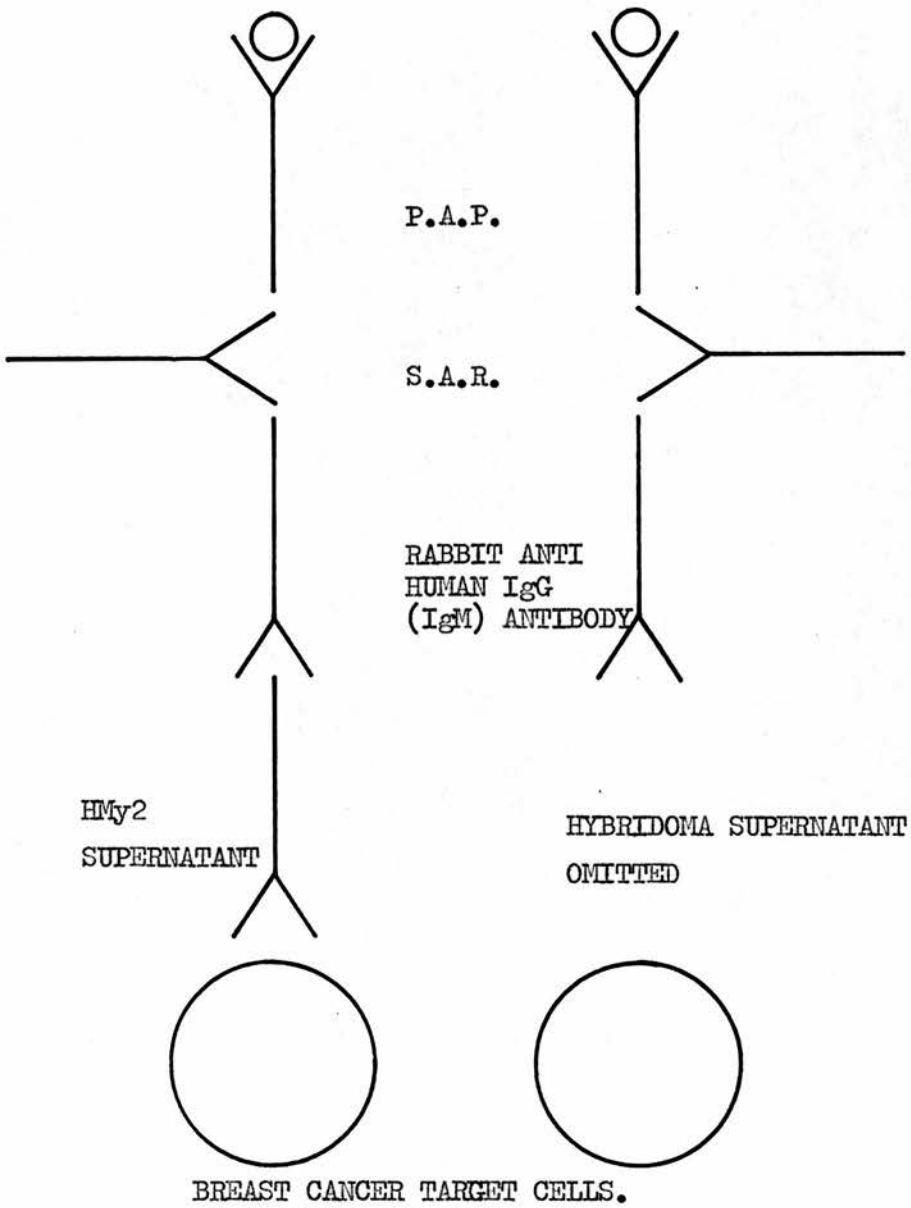


FIGURE 4.

THE IMMUNOPEROXIDASE REACTION.

THE NEGATIVE CONTROL EXPERIMENTS.



b) Immunoperoxidase-Results.

Each supernatant was tested against a variety of target cells as represented on table 25and 26. On this table the numbers in parenthesis represents the number of times that that supernatant was tested against that type of target cell-eg if (8) appears under the "other tumours" column this meant that the supernatant was tested against tumour sections from 8 different patients. Supernatants would be tested against numerous sections of each tumour . Similiarly (3) under the "own tumour" column meant that the supernatant was tested against sections of tumour from the patient from which the hybrid arose on three separate occasions. There was considerable variation in the number of types of target cellsagainst which each supernatant was tested. FE1/2/B2 and 32/1/B4 were the most widely tested since these hybrids grew very well in culture and hence a large amount of hybridoma supernatant was available for testing. These were used, therefore,in the early experiments establishing the technique. Some supernatants were obtained only in small volume before the hybrid died (eg 4/3/C2) and hence the supernatants were only tested against a few sections. Similiarly the later supernatants eg 72/2/D2 and 72/2/D4 were obtained towards the end of the study and time did not permit extensive testing of their reactivity. All supernatants were tested where possible against sections of tumours from which they were derived except where there was inadequate supernatant (6/3/A2, 6/1/A3) or where sections of the appropriate tumour were unavailable(5/1/D5,5/2/D5). Whilst 1/4/C2 and 4/1/B2 showed some degree of positive binding with an Fc rosette

preparation of some tumour cells this effect could not be repeated and was not maintained throughout testing against a variety of other cell types . This did not therefore seem to represent true specific binding to breast cancer cells.

Whilst a variety of binding patterns was obtained in many sections,none of these could be differentiated from the binding obtained from the appropriate negative control. It is therefore concluded that using the immunoperoxidase method no selective binding of McAb to breast carcinoma cells was demonstrated. Each of the supernatants were then concentrated X50 in a Minicon protein concentrator and re-tested against sections of their own tumours, but this also failed to reveal any selective binding of McAbs to breast carcinoma cells. Some examples of the background binding patterns obtained are shown in plates 2 and 3.

TABLE 25

Immunoperoxidase Stain.

Summary of the Results of Testing the Hybridoma Supernatants Against a Variety of Target Antigens.

1) Cytocentrifuge Preparations of Cultured Cell Lines

Hybrid.	Target Antigen			
	MDA	MCF 7	CLA4	DET 6
1/2/B2	(5)-ve	(5)-ve	(4)-ve	(4)-ve
1/4/C2	(2)-ve	(1)-ve	*	*
1/4/B6	(1)-ve	(1)-ve	(1)-ve	(1)-ve
4/1/A4	(2)-ve	(1)-ve	*	*
4/1/B2	(2)-ve	(1)+ve	*	*
4/1/C6	(1)-ve	*	*	*
4/2/C2	(1)-ve	*	*	*
4/3/C2	(1)-ve	*	*	*
5/1/D5	(1)-ve	(1)-ve	*	*
5/2/D5	(1)-ve	(1)-ve	*	*
6/1/A2	(1)-ve	(1)-ve	*	*
6/1/A3	(1)-ve	(1)-ve	*	*
6/3/A2	(1)-ve	*	*	*
6/4/A3	(1)-ve	(1)-ve	*	*
32/1/B4	(5)-ve	(5)-ve	(3)-ve	(3)-ve
53/3/A2	(2)-ve	(1)-ve	*	*
53/4/D4	(1)-ve	*	*	*
54/4/D4	(1)-ve	*	*	*
71/2/A1	(1)-ve	*	*	*
71/2/A2	(1)-ve	*	*	*

* = Not Performed.

TABLE 26.

Immunoperoxidase Stain

Summary of the Results of Testing the Hybridoma Supernatants Against a Variety of Target Antigens.

2)Preparations Derived from Breast Tumours

a)Cytocentrifuge preparations of Tumour Cell Suspensions (C.B.T.) and b) Paraffin Sections of Breast Tumours (P.B.T.)

Hybridoma	C.B.T.	C.B.T. + Fc Rosette	P.B.T. Own Tumour	P.B.T. Other Tumour
1/2/B2	*	(2)-ve	(1)-ve	(10)-ve
1/4/C2	(1)-ve	(2)+ve	(1)-ve	(1)-ve
1/4/B6	(1)-ve	(2)-ve	(1)-ve	(2)-ve
4/1/A4	*	(2)-ve	(1)-ve	(2)-ve
4/1/B2	*	(2)+ve	(1)-ve	(2)-ve
4/1/C6	*	(2)-ve	(1)-ve	(1)-ve
4/2/C2	*	(2)-ve	(1)-ve	(1)-ve
5/2/D5	*	(1)-ve	*	(1)-ve
5/2/D5	*	(2)-ve	*	(2)-ve
6/1/A2	*	(2)-ve	(1)-ve	(3)-ve
6/1/A3	*	(2)-ve	(1)-ve	(2)-ve
6/3/A2	*	(1)-ve	*	(1)weak+ve
6/4/A3	*	(2)-ve	*	(2)-ve
32/1/B4	(3)-ve	(7)-ve	(2)-ve	(13)-ve
53/3/A2	*	(2)-ve	(1)-ve	(7)-ve
53/4/D4	*	(2)-ve	(1)-ve	(5)-ve
54/4/D4	*	(2)-ve	(1)-ve	(5)-ve

71/2/A1	*	*	(1)-ve	(1)-ve
71/2/A2	*	*	(1)-ve	(1)-ve
74/2/D2	*	*	(1)-ve	(1)-ve
74/2/D4	*	*	(1)-ve	(1)-ve

* = not performed

PLATE 2

Immunoperoxidase Technique. Negative Controls.

Tissue section of a breast carcinoma in which the hybridoma supernatant stage of the assay was omitted. Staining of individual cells is demonstrated. These are thought to represent SIg bearing macrophages.

(Magnification x100)

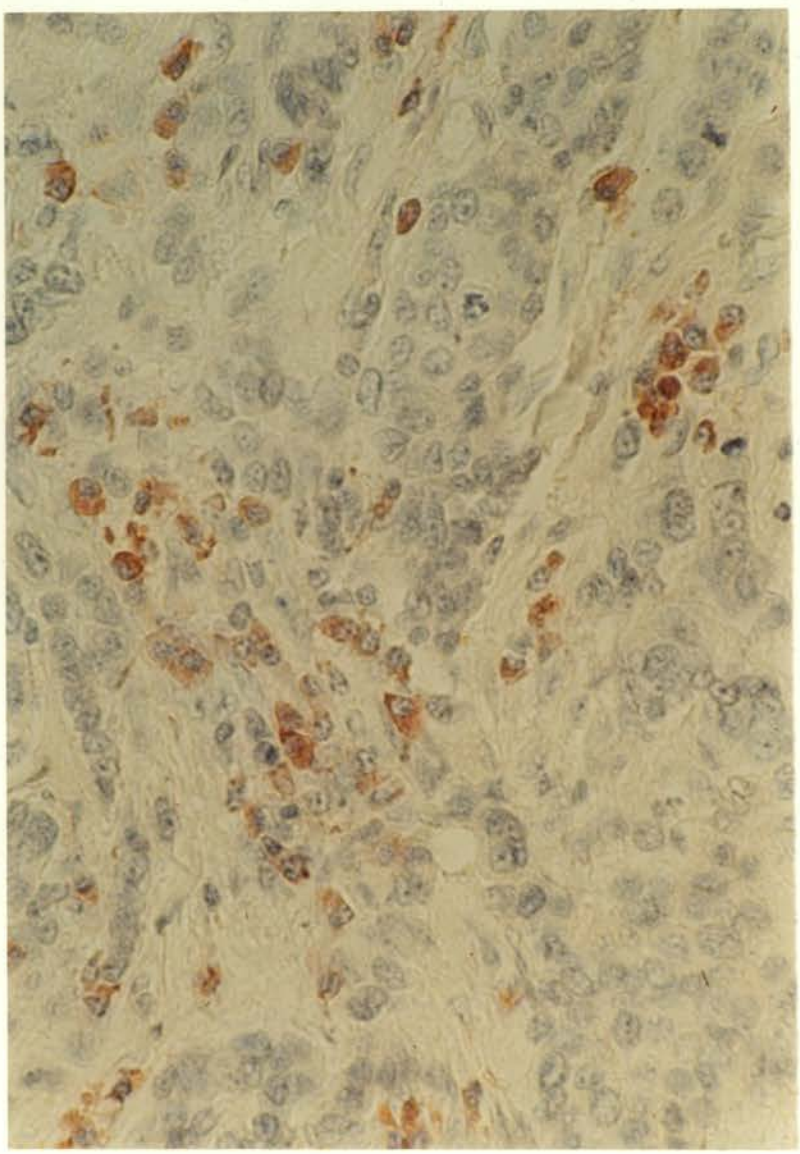
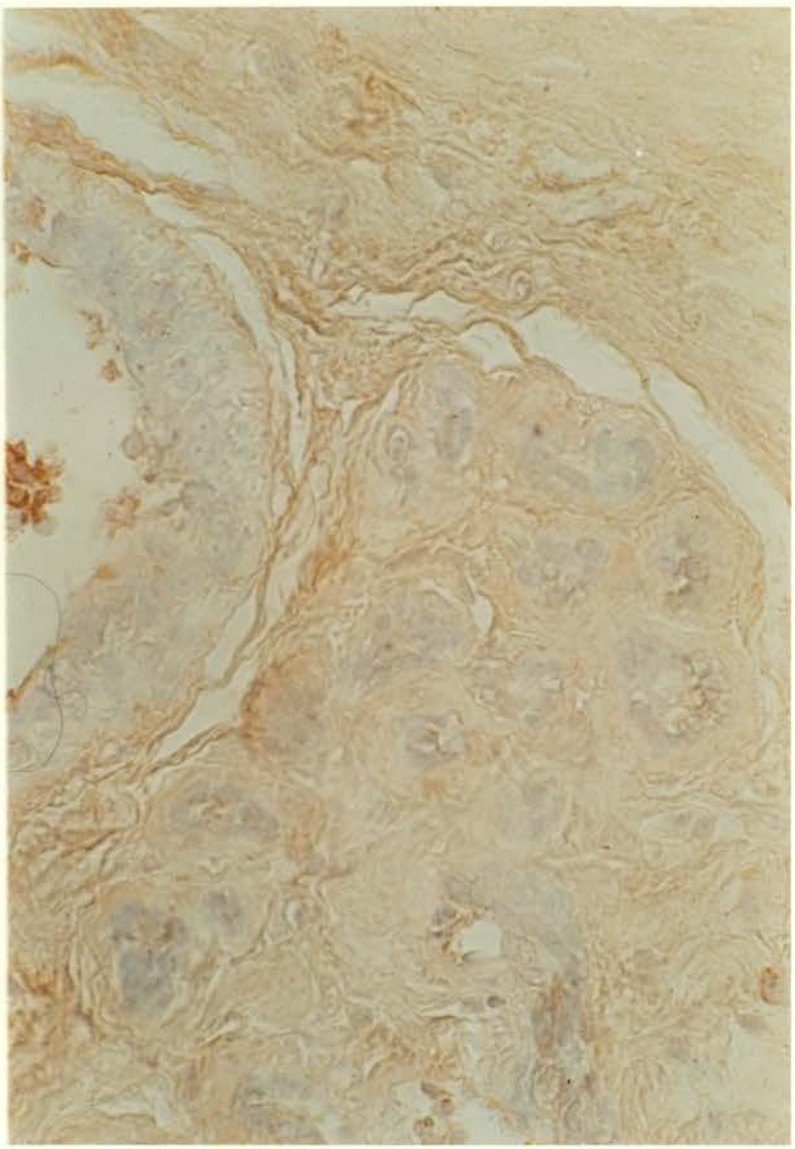


PLATE 3.

Immunoperoxidase Technique. Negative Controls.

Tissue section of breast carcinoma in which the hybridoma supernatant stage has been omitted. Staining of the fibrous connective tissue is demonstrated.

(magnification x100)



4.11. Cloning.

Cloning of hybrids was performed by limiting dilution as already described. Using this technique it was found that hybrids diluted down to one cell per well could be grown successfully up to large scale culture again in approximately 14 days. All hybrids which were growing well at the time of cloning were found to behave in this way. Similiarly ,those clones which were re-cloned were found to clone equally well when this was performed repeatedly. Hybrids grown from fusion of the HMy2 cell line with axillary lymphocytes clone well by limiting dilution which represents one advantage of this cell line when used for hybrid production.

Hybrids which were found by agglutination reaction to secrete both IgG and IgM (1/4/C2, 1/4/B6, 4/2/C2, 4/1/B2, 32/1/B4) were cloned repeatedly to determine whether it was possible to obtain two different clones, one secreting IgG and the other IgM or whether it was one hybrid which was producing both classes of immunoglobulin. These hybrids were cloned six times, and on each occasion clones were grown from the one cell per well dilution. These were then grown up in culture, had their supernatant tested and were then re-cloned. It was found that even after this procedure the hybrids secreted both classes of immunoglobulin. It is concluded that these hybrids were truly monoclonal from the outset, and were capable of secreting both IgG and IgM concurrently.

SECTION 5.

DISCUSSION OF EXPERIMENTAL WORK.

The following section will critically discuss the experimental work described in this thesis and relate it to published data in the literature.

5.1. Materials and Methods.

5.1.1.Lymphocytes.

Successful cell fusion requires as one of the parents a lymphocyte sensitised by the appropriate antigen. The spleens of immunised mice provide a ready source of sensitised lymphocytes. Human McAb production requires a source of sensitised human lymphocytes.

One possibility as suggested by Reading (1982) is to sensitise lymphocytes in vitro. Whilst the techniques for this are described,and may be technically feasible for some antigens it has disadvantages in the production of anti-tumour antibodies. This is because the characterisation of tumour-specific antigens remains incomplete, and hence it would be difficult to know what antigens to use in these circumstances. The use of lymphocytes sensitised in vivo appears to be a more suitable choice.

Peripheral blood lymphocytes are the most easily obtained source of lymphocytes but according to Eremin et al.(1976) there was found to be little difference in the lymphocyte subgroups in the peripheral blood of normal and breast cancer patients, as assessed by a broad group of phenotypic markers (T,B,Fc,C3), suggesting that peripheral blood lymphocytes were not greatly affected by any tumour-host immune reaction. Even if blood lymphocytes contained tumour-sensitised cells, it would be

difficult to separate these out from the other non-allergised cells. Thus lymphocytes from tumour draining nodes or infiltrating the tumour itself would appear to provide a more useful source in spite of their relative inaccessability. Whilst anti-glioma McAbs have been prepared using tumour infiltrating lymphocytes (Sikora et al.,1982(b)) the use of lymphocytes infiltrating breast tumours for cell fusion was not attempted in this study. This was because it was found that the techniques employed to isolate viable lymphocytes from specimens of breast cancers resulted in an inadequate number of lymphocytes for cell fusion (Eremin, personal communication).

Axillary nodes on the other hand ,are a suitable source of lymphocytes for cell fusion work,since they are able to provide large numbers of lymphocytes some of which may have been sensitised by tumour associated antigens. Other workers (Schlom et al 1980, Sikora 1982(a)) used a similiar strategy. Having accepted that axillary nodes may be a suitable source of lymphocytes it is important to ensure that these lymphocytes are in an optimal biological state for cell fusion.

Two aspects of the preparation of axillary node lymphocytes require consideration. Firstly, only sensitised B-cells are required for fusion, although lymphocytes obtained from the axillary node will contain a mixture of B and T lymphocytes. It may be advantageous to try to select out the sensitised B lymphocytes prior to fusion, a point emphasised

by Sikora et al.1982(c). Whilst it may be very difficult to isolate the sensitised B lymphocytes from the rest of the B lymphocytes it may be possible ,and probably desirable,to separate the B from the T lymphocytes. The latter may be done in a variety of ways. Anderson et al. (1983) described a method whereby B lymphocytes were isolated by a process of immune rosette depletion. Using this technique,T-cell rosettes were made and separated from the lymphocyte suspension. The disadvantage of the technique is that often small numbers of viable B-cells only are obtained at the end of the procedure. This technique has been used successfully to prepare enriched B cell populations prior to fusion (Gigliotti et al.,1984, Littman et al.,1983, Larrick et al.,1983).

A different approach has been to use a B-cell mitogen in the mixed lymphocyte culture, in order to expand the B-cell pool. This approach has the advantage that it will also fulfill the second requirement of lymphocyte preparation namely, that lymphocytes are actively growing and dividing at the time of fusion. As has been discussed in the first section of this thesis successful fusion requires the presence of two actively dividing cells for nuclear fusion to occur. This requirement has been emphasised by Goding(1980). A variety of mitogens may be used in this respect and in this study pokeweed mitogen was used. Pokeweed mitogen (PWM), a plant lectin,is described as a specific B cell mitogen,

although this effect may be dependent on the presence of T cells (Keightley et al.,1976, Hammarstrom et al.,1979). It has been used by other workers to improve the rate of human hybrid production by stimulation of both peripheral blood lymphocytes (Ritts et al.,1983 Shoenfield et al.,1982, Olsson et al.,1983)and node lymphocytes (Warenius et al.,1983),and would appear to be an appropriate mitogen to use.

There are other methods by which B cells may be stimulated and these perhaps merit further investigation. Kearney et al (1975) described the way in which bacterial lipopolysaccharide non-specifically stimulated B lymphocytes and increased immunoglobulin secretion by these cells. Lamers et al (1982) discussed how little is known about the mechanisms by which B-cells are stimulated to proliferate. A murine McAb is described which bound to the IgG Fc surface receptor on the B lymphocyte and stimulated B cell proliferation and immunoglobulin secretion.It was postulated that this McAb acted in a similiar manner to lipopolysaccharide.

Another experimental approach is to infect the lymphocyte suspension with Epstein Barr virus (EBV). EBV has been shown to specifically infect human B cells (Epstein et al.,1982) and to transform them into established cell lines. It was postulated (Steinitz et al.,1979)that all B lymphocytes possessed EBV receptor sites and when infected were transformed into proliferating cell lines.B lymphocytes thus transformed did not have the ability to secrete the virus nor to transform other cell lines. (Epstein et al 1982). EBV transformation

has also been shown to increase the rate of immunoglobulin secretion by the transformed cell (Rosen et al., 1977) . The techniques of EBV transformation of lymphocytes is relatively simple. The virus is secreted by the marmoset blood leucocyte line B95-8 which can be grown readily in culture (Miller et 1973). The supernatant of this culture, containing EBV , can be collected frozen,and stored until required for use. Lymphocytes can then be transformed by incubating them for one hour at 37°C in the presence of the EBV containing supernatant (Steinitz et al., 1979). The resulting transformed B cells can then be cloned, grown in culture and have their secreted immunoglobulin measured. This technique has been used per se to produce human McAbs. Steinitz et al (1979) demonstrated the feasibility of this by transforming peripheral blood B lymphocytes of patients with high natural titre against nitrosonitrophenol. By cloning the resulting transformed cells a cell line was obtained which produced immunoglobulin with high anti-NNP activity. Slaughter et al.(1978) and Steinitz (1980) both used this technique to tranform peripheral blood lymphocytes from patients with rheumatoid arthritis in an attempt to produce monoclonal rheumatoid factor. This technique proved to be successful in their hands. Boylston et al.(1980) used EBV transformation of peripheral blood lymphocytes to produce anti-D antibodies. Chiorazzo et al.(1982) used EBV transformation of enzyme deficient B-cells to provide the myeloma partner for cell fusion.

Watson,D.B., et al, (1983) transformed tumour infiltrating lymphocytes from melanoma tumours with EBV and these produced antibody with some activity against human melanoma cells. Steinitz et al .(1984) produced an anti-pneumococcal IgM by EB virus transformation of peripheral blood lymphocytes of immune volunteers. This technique appears to have the advantage of allowing B cells to selectively grow and secrete antibody. Indeed at first sight it appears to be a much simpler way of producing monoclonal antibodies than the somewhat complex techniques involved in cell fusion. It does however have disadvantages.

The transformed B cell cultures, whilst providing proliferating cultures in the early stages have been found to die after several months in culture, which would be disadvantageous since supplies of any useful antibody would be limited. (James et al 1984, Kozbor et al.,1982).In addition the transformed lymphocytes secrete lower concentrations of immunoglobulin than the comparable hybridomas.

The technique may be of considerable value if the transformed B cells are subsequently fused to an appropriate myeloma line, since the myeloma parent would hopefully increase the rate of immunoglobulin secretion and also make the B cell stable in culture. Kozbor et al,(1982) fused EBV transfomed lymphocytes with a variant of the human myeloma cell line GM1500. This variant was selected for resistance to oubain. The resulting hybrids were grown in medium containing oubain as well as HAT so that the parent transformed lymphocytes would die in culture.Using this technique antibody secreting hybridomas were obtained which grew better in culture than the original transformed lymphocytes.

Thus EB virus appears to be a very useful way to select B cells out of the lymphocyte suspension ,and to induce these cells to grow and proliferate prior to fusion. It may even be possible to select by repeated screening and cloning of the transformed lymphocytes those lymphocytes which secrete immunoglobulin reactive with the appropriate antigen prior to fusion. Kozbor et al.(1984) described the use of EBV transformed peripheral blood lymphocytes in fusion experiments to produce human monoclonal anti-tetanus toxin antibody .These lymphocytes were selected out on the basis of the activity of their secreted immunoglobulin prior to fusion.In the latter study it was reported that the transformation of peripheral blood lymphocytes prior to fusion produces six times more hybrids than lymphocytes stimulated with PWM and thirty six times more hybrids than unstimulated lymphocytes. These advantages were obtained at the cost of introducing further complexity into the fusion system by using oubain. It has been found to be relatively easy to obtain an oubain resistant variant of the HMy2 cell line (T.Alderson,personal communication) and these techniques would appear to be applicable to the fusion system used in this study. It is hoped that the merits of EB virus transformation may be investigated at some time in the laboratory in which this work was carried out.

It is of interest that the importance of prestimulation of lymphocytes prior to fusion has only been mentioned in respect to human-human hybridoma production.This reflects the sources of

human lymphocytes used for hybrid production. In murine McAb production the spleens of immunised animals were used as the source of lymphocytes. Spleens were removed from these animals after strong antigenic stimulation and the spleen cells obtained from these animals have been found to be actively dividing at that time (Andersson et al 1978).Therefore the lymphocytes obtained from these spleens are in optimal condition for fusing.

Human lymphocytes used for hybrid production are likely to have a much lower rate of cell division than the spleen cells of immunised mice. Peripheral blood lymphocytes have a very low rate of mitotic activity whilst node lymphocytes may have some degree of activity especially if the node is being challenged by the appropriate antigen. One would expect that the higher the rate of cell division,the greater would be the success rate for obtaining hybridomas from these lymphocytes .This is confirmed by some comparisons of the rates of hybrid production compared with the source of human lymphocytes used.

Olsson et al, (1983) found a lower hybrid rate using peripheral blood lymphocytes compared with spleen cells. Glassy et al.(1983) compared the rates of hybridoma production obtained by fusing human lymphocytes of various types with the myeloma cell line UC 729-6. In the latter study peripheral blood lymphocytes were the least efficient hybrid producers, spleen cells and node lymphocytes less so, and tonsil lymphocytes were found to be most efficient cells to use. These spleen cells were obtained from people who had not received an antigenic challenge. Tonsil lymphocytes from children would be expected to have a high rate of cell proliferation since the tonsils are constantly undergoing antigenic stimulation.

Larrick et al.(1983) found tht B-cell blast fused much more readily than unstimulated B cells. Thus it appears that the rate of hybrid production is in part determined by the degree of proliferation occurring in the parent lymphocyte at the time of cell fusion. This may be one of the main reasons why murine McAbs are much easier to produce than autologous human McAbs and further investigation is required to define the optimum regimes for stimulating various types of human B-cells to grow and divide prior to fusion.

Finally Olsson et al.(1983) maintained lymphocytes in HAT medium during prestimulation with PWM, to induce the enzymes necessary for the alternative route for purine/pyrimidine synthesis prior to fusion. This was not done in this study but it may be an important point of technique and merits further investigation.

5.1.2. The Myeloma Cell Line.

The HMy2 cell line was kindly donated to the laboratory by the Ludvig Institute for Cancer Research in Cambridge. The development of the HMy2 line from the ARH 77 plasma leukaemia cell line has been described (Edwards et al.,1982). The ARH 77 line was derived from a patient with plasma cell leukaemia as described by Burk et al.(1978). The HMy2 line has been found to possess the Epstein-Barr nuclear antigen suggesting that it may represent an EBV transformed cell line. The HMy2 line has been found however to neither secrete the virus nor transform other cells. (Edwards et al.,1982). Throughout the course of the study it was found to grow reliably and readily in culture and to freeze and thaw with little difficulty. Periodically (every 10 passages) subcultures of the HMy2 line were grown in HAT medium, to ensure that there were no spontaneous revertants of the HPRT deficient cells. Every time this was performed the HMy2 cells died in HAT medium indicating that no spontaneous reversion had occurred. This same finding has been reported with other human myeloma cell lines (Ritts et al., 1983, Glassy et al.,1983)and also with murine myeloma cell lines. suggesting that clones of myeloma cells selected for resistance to 8 azaguanine are stable mutants and spontaneous reversion is a rare occurrence.

Reading (1982) mentioned the problem of mycoplasma infection in these established cell lines. Whilst the HMy2 cell line was screened for mycoplasma infection prior to the commencement of this study, routine testing of the HMy2 line and resulting

hybrids for mycoplasma infection was not performed during this study. The HMy2 cell line does possess the disadvantage that it secretes an immunoglobulin (IgG kappa chain) (Edwards et al., 1982). One advantage of the line , on the other hand,is that it has been grown in serum-free medium which is an advantage when analysing the secreted products of resulting hybrids . Growth in serum free medium was not attempted in the course of this study but would have been had a McAb been obtained which reacted specifically with breast cancer cells.

The HMy2 line has been found to produce hybrids consistently in a number of different laboratories. (Warenius et al.,1983;Cote et al.,1983). As has been pointed out (Sikora et al 1982(c))this is a feature not shared by some other cell lines.

Several other human myeloma/plasmacytoma cell lines have been described for use in the production of human hybridomas. Olsson et al.(1980) described an 8 azaguanine resistant mutant of the U266 myeloma cell line. This cell line secreted an IgE, was used successfully in cell fusions and given the title SKO-007. Croce et al.(1980(a)) employed an HPRT deficient variant of the GM1500 myeloma cell line which secreted an IgG. A subline of this ,GM 4672 was used successfully by Shoenfield et al.(1982) to produce human hybrids. Kozbor et al.(1982) successfully developed an oubain resistant variant of this line and fused it with EB virus transformed lymphocytes. The relative efficiencies of the SKO 007, HMy2 and a murine non secreting cell line were compared as fusion partners for human lymphocytes by Cote et al.(1983) who concluded

that the HMy2 cell line compared favourably with the other human myeloma cell lines but the murine non-secreting myeloma cell line was the more efficient hybrid producer. Olsson et al.(1983) compared the properties of the SKO 007,RH L4 and a murine nonsecreting line. The RH-L4 line was found to produce a higher percentage of hybrids than the SKO-007 line. It was also found that the rate of hybrid production,produced by the mouse myeloma line was of the same order as the human lines when peripheral blood lymphocytes were used as fusion partner, although the hybridoma production rate of the mouse myeloma line with mouse spleen cells was much higher. Glassy et al,(1983) have reported a different myeloma cell line ,U729-6 which is said to form stable hybrids. This cell line exhibits surface IgM . Experience with this cell line is as yet limited. There have been two reports of human non-secreting myeloma cell lines useful for fusion. Pickering et al,(1982(a)) described an 8-azaguanine resistant variant of the RPMI 8226 myeloma cell line which was described as forming stable hybrids when fused to human lymphocytes. This cell line was said to lack surface Ig and did not secrete immunoglobulin. Unfortunately, this line was later shown to be of murine and not human origin(Pickering et al. 1982(b)).Ritts et al. (1983) described a nonsecreting human plasmacytoid cell line which had been shown to possess intracytoplasmic IgG but to have no SIg or secreted imunoglobulin. This line has been shown to form hybridomas successfully with human lymphocytyes.

Further reports about this cell line are awaited to assess its efficiency for use in hybrid production.

Abram et al. (1983) compared six human myeloma/plasmacytoma cell lines and one mouse cell line . The human cell lines used were U 266, RPMI 8226, HMy2, U729-6, HF 2, GM 4672. These were compared for rate of hybrid production, level of immunoglobulin secretion, and stability of hybrids. HMy2 was found to produce a higher percentage of hybrids, and more stable hybrids than the other lines although these hybrids secreted slightly lower levels of immunoglobulin.

In conclusion,there is no universally accepted "ideal" human myeloma cell line for use in cell fusions. Olsson (1985) reported that there are, as yet, only 15 human myeloma / lymphoblastoid cell lines freely available for cell fusion. Such a line would require to be a nonsecretor of immunoglobulin, produce a high rate of hybridoma formation, and a high level of antibody secretion by these hybridomas.In the absence of such a line HMy2 was regarded as a suitable choice for use in this study in spite of its limitations.

5.1.3. The Fusion Experiment

There appear to be many variations in the way in which fusion experiments are performed (James et al.,1984) a fact which by itself suggests that more study is required to evaluate properly the various methods in use so that the optimum method for fusing cells may be established. It has been said (Fazekas 1980(b)) that since the introduction of McAbs by Kohler and Milstein (Kohler et al.,1975,1976) relatively little attention has been paid to the mechanisms by which hybridomas are produced, since most workers have aimed to produce as many McAbs as possible. Whilst this is understandable, there is a need for the fusion experiment to be more completely investigated. In this section the fusion experiment will be discussed to try to determine those areas in which more study is required.

Choice of Fusogen.

Whilst the initial work of Kohler and Milstein and others (Yerganian et al.,1966, Harris et al.,1965) used Sendai virus as the fusogen of choice,there is now wide agreement that polyethylene glycol (PEG) ,described by Pontecervo (1975), is the fusogen of choice (Galfre et al.,1977). There are a variety of ways in which PEG is used and it is interesting in this context to study Pontecervo's description of the effects of PEG.

In his work the cells to be fused were first washed and suspended in serum free solution. It is implied that serum inhibits the activity of PEG although the mechanism of this is unknown. This point is emphasised by Sikora et al (1983(a)) and hence all fusions in this study were performed in the absence of serum.The strength of the PEG solution and the duration of exposure also varies. Pontecervo layered on a solution of 10g of PEG in 10 ml of solution for 5-15 minutes. Olsson et al (1980)0 used 38% PEG, whilst Edwards et al (1982) used 1 ml of a 50% solution of 1500 PEG in 1-15 medium.

Pontecervo observed that following the addition of PEG in this manner considerable shrinkage of the cells occurred. PEG exerts a strong osmotic effect on the cells which may be potentially damaging . For this reason Fazekas et al (1980(a)) advocated the addition of DMSO (dimethyl sulphoxide) to the PEG solution to prevent osmotic damage. DMSO was added to the first of the two PEG solutions used in this study although it is not known whether this protection against osmotic damage is more important than the potentially toxic effects of the DMSO on the growing hybrids. Osmotic damage may occur particularly when the PEG is removed and the cells rehydrated. Pontecervo obsered that rapid dilution at this stage killed the cells. It therefore seems sensible to use a second more dilute PEG solution before adding medium since this will protect against rapid dilution of the cells, as described by Sikora et al (1983(a)). This practice was adopted throughout this study.

Relatively little attention has been paid to the temperature and PH of the fusion mixture. White et al. (1980) emphasised the importance of a slightly acid pH when Sendai virus was used as fusogen. Whilst it is thought that PEG exerts its optimal effect at a slightly alkaline pH there is no definite evidence of what is the optimum pH. In this study the precise pH of the PEG solutions were not measured although they were made slightly alkaline as judged by the colour of the culture medium in which it was disolved. Likewise while most authors suggest a temperature of 37°C for cell fusion (eg Edwards et al.,1982) there is little evidence to show the importance of a particular temperature for cell fusion. The maintenance of constant temperature in the fusion experiments in this study was not carried out. Whilst all solutions of PEG and medium were maintained at 37°C prior to fusion, the cell suspension and solutions were not maintained in a 37°C waterbath throughout the fusion experiment.Fluctuations of temperature obviously occurred towards the end of the experiments. This may in part explain the poor hybrid rate obtained in this study.

Management after the Addition of PEG.

Most studies agree on the need to dilute the PEG very gently to avoid osmotic damage to the cells. In this study, care was taken to add the first 4ml of medium dropwise, after which the cell suspension was left to stand for 3-4 min. Following this the next 29ml of medium were again added dropwise. Finally the cell suspension was very gently aspirated into a wide bore 25ml pipette. Sikora et al,(1983(a)) is one of the few to mention this detail.

Following cell fusion the next point of debate is how to dispose of the PEG. In this there is a conflict of interests. On the one hand it is undesirable to leave the fused cells too long in PEG since this may inhibit hybrid growth, on the other, the newly fused cells are still very fragile, held together only by the adhesion of the cell membranes. Over vigorous treatment at this stage may disrupt this bond.Pontecervo performed the fusion experiment in a monolayer culture of cells and removed the PEG by "draining it off". Thus he achieved both aims-to remove the PEG and not to disrupt the cells. Cell suspensions, however,are not amenable to this treatment. Many workers have removed the PEG by centrifuging the cell suspension immediately after fusion and resuspending the cells in medium (eg Pickering et al 1982(a), Glassy et al 1983, Warenius et al 1983). Olsson et al (1980) even washed the newly fused cells before plating them out.

The alternative means of removing PEG is to dilute the PEG out of the medium by successive changes of medium. This is the technique described by Sikora et al.,(1983(a)) and used in this study. Following fusion the 25 ml cell suspension was dispensed into two 24 well plates. Each well was then fed with 1.0ml of fresh medium.Thus the original 2.0 ml of PEG solution was diluted approximately 1:47 within 10 min. of being added to the cell suspension. On the day following fusion one ml of medium was removed and replaced with fresh medium. This process was repeated daily for 7 days. It was assumed that by using these techniques

the amount of PEG left in contact with the hybridomas was negligible. It is unknown however whether the trauma of centrifugation is more harmful to the young hybrids than the prolonged exposure to small amounts of PEG and this requires further investigation.

d) Feeding and Selective Medium.

The care of hybrids after fusion varies from study to study, in particular the frequency with which medium changes are made and the time at which selective medium is added .All authors agree on the need to change the medium in the culture wells from time to time but the frequency varies. This is an important point to establish since each medium change means that the plates are exposed to the possibility of infection (Fazekas 1980(b)). The purposes of medium changes appear to be threefold.Firstly fresh medium provides a source of nutrients for the growing cells. In the experience of this study this need appeared to occur at two times during the course of the culture,these being at the beginning before all the parent cells die , and at the end when hybridomas were growing briskly and required frequent feeding. In between these two episodes in the natural history of hybridoma formation there is a spell when little cell growth is evidentin the wells and the need for very frequent medium changes at this time is debatable.

Secondly medium changes provide a means of removing the potentially harmful remains of the dead cells. The occurrence of cell fusion has been described as being a relatively rare event even using PEG. Goding (1980) estimated that fusion between cells occured approximately once amongst 10^6 cells. Hence, there will be

a lot of cell debris in the well which may inhibit the growth of young hybrids. On these grounds fairly regular medium changes may be required,especially early in the study when most cell death occurs.

Thirdly medium changes provide a means for removing PEG from the medium if the cells are not centrifuged after fusion,which means that frequent feeding in the first few days is important.

For these reasons a feeding regime was used which involved frequent medium changes early and late in the life of the plate, whilst intermittent rates of feeding were carried out in the intermediate phase of hybrid growth. There is a large variation in the feeding regimes used in different studies from daily (Wunderlich et al.,1981) to weekly (Ritts et al.,1983) to only two medium changes in the life of the plate (Kennett et al.,1978). There is a need for a study of the relative beneficial effects of these regimes.

The addition of selective medium is an important step in selecting out hybridomas, and there are different methods described in doing this. In this study selective medium was added 24 hours after fusion. The reasoning behind this was based on the presumed pattern of fusion in the emerging hybrid. It was assumed as described by White et al. (1980) that the initial fusion event involved only the fusion of the two cell membranes and that nuclear fusion did not occur until the next stage of cell division. It seemed , therefore that fusion would not be

complete until several hours after the fusion experiment was performed . Since the survival of hybrids depended upon the genetic properties of both cells it will take some time before cellular metabolism is controlled by the fused nucleus ,and hence it was reasonable not to add the selective medium until this has occurred. To add selective medium at the time of fusion (Fazekas et al.,1980(a)) may inhibit division of the myeloma parent and hence prevent fusion occurring.Other studies have likewise delayed adding selective medium until 24 hours after fusion (Astaldi 1980). One possible further improvement to this regime was sugested by Cote et al.(1983) who added Hypoxanthine and Thymidine at the time of fusion and Aminopterin at day 1. This would provide the stimulus for the lymphocyte parent to adopt the alternative pathway of purine/pyrimidine synthesis whilst not inhibiting the myeloma cell. Equally it may be beneficial to keep the lymphocytes in HT medium prior to fusion as already mentioned

In conclusion the techniques used in this study have a sound theoretical basis, but much of this has not been tested by controlled trial. As the number of laboratories working on McAb production is increasing rapidly, there is a need for more careful evaluation of the various steps involved in the fusion process.

5.1.4. Foetal Calf Serum.

Hybrids were grown in medium, containing foetal calf serum (Gibco).The importance of testing serum for its efficiency in producing cell growth has been emphasised (Cote et al.,1983)) since the efficiency of serum has been found to vary widely. Edwards et al.(1982) tested each batch of serum for its cloning efficiency and its ability to maitain adequate growth of a HMy2 culture. In this study each batch of serum was tested for its ability to support vigorous growth of the stock culture of HMy2 and also for the growth of whatever hybrids were in culture at that time. Only one batch sample was found to be inadequate in the course of this study.

Growing hybridomas in foetal calf serum presents three potential problems. Firstly,it is expensive,and may be decreasing in availability (Anderson 1980) both factors which will increase the production costs of any McAb being prepared commercially in large scale culture. Secondly, the presence of serum in the supernatant of hybridomas may complicate the characterisation of these supernatants. Thirdly, if a McAB is being prepared for clinical use it is highly undesirable to have foreign proteins mixed in with the McAb and separation of the two may prove difficult. For these reasons it may be of value to grow hybrids in serum free medium as advocated by Goding (1980) although Wunderlich et al. (1981) found that immunoglobulin secretion by hybridomas decreased when this was done. A variety of such media are available eg Iscoves medium (Iscove et al.,1978) and attempts have been made to identify the essential growth factors required in such medium

(Murakami et al,, 1982). The HMy2 cell line and resulting hybrids have been grown in serum free medium in other laboratories as already described and the method is feasible. This may be of great value when producing McAbs for clinical use.

5.1.5. Screening of Supernatants.

The screening of supernatants has two distinct roles.The first of these is to determine the type and concentration of immunoglobulin secreted by the hybridoma and the second is to determine the activity of any McAb produced with the appropriate antigen. Part of this study was aimed at developing suitable methodology for the screening of human McAbs for reactivity against breast cancer cells, which were appropriate both to the type of antigen and to the facilities of the laboratory. The need to have both an accurate and reliable method for the screening of hybridoma supernatants is emphasised by Goding (1980). There are a large variety of techniques described for screening McAbs in each of the above two areas and these are now discussed in turn and their relative merits compared.

5.1.6. Estimation of Antibody Type and Concentration.

The aim of this part of the screening process was to identify the type and class of immunoglobulin secreted by the hybridoma. This is important since it will show whether the hybridoma is secreting merely the myeloma immunoglobulin or whether it is producing a new immunoglobulin derived from the lymphocyte parent.(assuming the

myeloma is an immunoglobulin secretor.) Since both myeloma and lymphocyte may secrete the same class of immunoglobulin ,analysis of the light and heavy chains is often required. Knowledge of the type of antibody secreted will allow the use of appropriate reagents when assessing the reactivity of the McAbs to the target antigens. Knowledge of the concentration of antibody produced is important since it allows comparison of the relative merits of the various myeloma cell lines and also gives some indication of the scale of operation that would be required to produce a McAb commercially.A variety of mechanisms have been used to determine the type,class and concentration of MAbs. Underlying these methods are two distinct principles.

The first involves the use of specific antibodies with reactivity against different classes of immunoglobulin, and individual light and heavy chains, whilst the second involves analysis of the immunoglobulin chains on the basis of their physico-chemical properties. The use of specific antibody against the immunoglobulin being tested relies on the fact that there is a method by which sites of antibody binding can be recognised by an appropriate indicator , such as a radioactive tracer or an attached sheep red blood cell. Use of a radioactive tracer in this context has been widely used in a radioimmunoassay to identify the characteristics of any McAb present. Olsson et al,(1980) used I^{125} as the tracer and tested for the presence of immunoglobulin in a radioimmunoasssay using Staphylococcal protein-A.

A similiar technique was adopted by Wunderlich et al. (1981).Staph.protein A has been shown to bind to certain subgroups of IgG (Kronvall et al.,1969, Goding,1978). This binding has been shown to occur at the Fc portion of the heavy chain, binding occurring to IgG subgroups 1,2 and 4 but not 3.(These subgroups identify the type of heavy chain present.) Schlom et al.,(1980) used a radioimmunoassay in which the supernatants of hybridomas were tested for the presence of antibody by their ability to bind onto rabbit anti-human IgG and IgM, which in turn was bound onto polyacrylamide microspheres. The amount of antibody present could then be estimated by competitive binding in a column with I^{125} labelled human IgG and IgM. Pickering et al. (1982(a)) described the use of the Ouchterlony double diffusion method to test for the presence of antibody against a variety of anti-immunoglobulin antibodies. Whilst the technique provided a relatively easy way of determining the type of antibody present , a radioimmunoassay was still required to measure the concentration of the antibody. Sikora et al. (1983(b)) described an radioimmunoassay for the detection of hybridoma antibody which was sensitive to a concentration of 20ug/l of immunoglobulin,emphasising how sensitive a technique the radioimmunoassay can be for measuring immunoglubulin concentrations.

An alternative means of labelling these antibodies is by an indirect red cell agglutination reaction. Claflin et al (1978) described the use of a simple and rapid means of

detecting immunoglobulin by using sheep red cells coupled with the appropriate antibody. Sikora et al. (1981) described the way in which Staph.Protein A can be used in conjunction with an SRBC lysis test to test for immunoglobulin production. Watson,D.B. et al. (1983) described the use of an agglutination reaction to measure the type and concentration of antibody present which was similiar to that used in this study. The concentration of antibody could be calculated by comparing the agglutination reaction pattern of a solution of known immunoglobulin concentration with the hybridoma supernatant. The concentration at which agglutination failed to occur should be the same in both instances and from this the concentration of antibody in the hybridoma supernatant could be calculated .

The two techniques are both widely used in the screening of hybridoma supernatants. The radioimmunoassay is a sensitve assay although background binding caused problems in this study. Its main disadvantages are that it is a somewhat lengthy procedure and is also expensive because of the use of the radioactive tracer. It also requires special equipment to count the radioactivity. These problems were emphasised by Claflin et al. (1978).The agglutination assay has the advantages that it is both quicker to perform and much cheaper. In the experience of this study the problems of false positive results did not occur provided that each batch of coupled red cells was properly tested with positive and negative controls before use. It is also a

sensitive assay. Using known concentrations of immunoglobulin, IgM produced an agglutination reaction to a dilution of 10^{-10} g/l and IgG to a dilution of 5.6×10^{-12} g/l . This compared favourably with the sensitivities reported for the radioimmunoassay. Thus the agglutination reaction seems to possess several advantages over the radioimmunoassay and in this study was found to be an accurate and reproducible means of measuring antibody type and concentration. The disadvantage of both the radioimmunoassay and the agglutination reaction concerns the antibody reagents used. Specific antibodies against the type and class of McAb are required for both assays and the specificity of these antibodies limits the accuracy of the assay. Having considered this limitation, the agglutination reaction still represents an easy, reliable and inexpensive assay for the screening of hybridoma supernatants for the presence of immunoglobulin. There is, however , a need for a more accurate way of defining the precise characteristics of any antibody secreted by hybridomas which does not rely on the specificity of other antibodies. A suitable technique for doing this is the technique of isoelectric focusing which involves the separation of complex mixtures of proteins such as immunoglobulin chains on the basis of their physico-chemical properties. The technique was described in some detail by Awdeh et al.,(1968).The distribution of molecules on the polyacrylamide gel was determined by their isoelectric focusing along a pH gradiant in a carrier ampholyte (sodium dodecylsulphate- hence the name of the technique is abbreviated to SDS-PAGE).

By this technique groups of immunoglobulin chains could be analysed accurately. If the hybrid was grown in medium containing C^{14} leucine, this label will be incorporated into the secreted McAbs, and hence when these McAbs undergo SDS-PAGE their positions on the polyacrylamide gel can be estimated by autoradioography on a photographic plate.

The technique of SDS-PAGE was further described by O'Farrell(1973,1975) who advocated it as a an accurate way of separating mixtures of proteins. Olsson et al (1983) used this technique as an initial screen of hybrid supernatants.

It is suggested therefore that the agglutination reaction is a suitable means of screening hybridoma supernatants for the presence of antibody. Subsequently if any of these antibodies demonstrate specific binding to the target antigen and thus merit further investigation, then SDS-PAGE should be applied at that stage to fully characterise the antibody. In addition the isotype of this immunoglobulin could be identified using specific antisera, or, where applicable, protein A affinity chromatography.

A criticism of this study was that it was not established whether the majority of McAbs produced contained antibody derived from the parent lymphocyte as well as the myeloma cell. SDS-PAGE of hybridoma supernatants can be of value in identifying the immunoglobulin chains secreted by the hybridoma,confirming its monoclonality and comparing the hybridoma chains with those secreted by the parent lymphocyte and myeloma cells.

5.1.7. Screening The Reactivity of McAbs with the Target Antigen.

A method is required to screen the reactivity of McAbs against the appropriate antigen, in this study breast carcinoma cells. The requirements for such an assay may be defined as follows.

It requires a high degree of specificity- that is it requires to be able to identify sites of McAb/antigen binding without either an excess of background staining or a poor detection rate of McAb/antigen binding sites. It is important therefore that appropriate negative and positive controls are included in the assay. The assay must also be sensitive -that is it must be able to detect very low concentrations of McAb/antigen binding.

In addition it is preferable that the results of the assay bear some relationship to the possible in vivo activity of the McAb.

Finally the assay should,if possible, be relatively inexpensive and easy to perform. A wide variety of techniques have been proposed for the screening of the reactivity of McAbs,their use depending on the nature of the antigen.The techniques available to determine sites of McAb binding to tumour cells were summarised by Sikora (1982(a)) as follows.

1) Radioimmunoassay.

2) Immunohistology.

3) Cytotoxicity tests.

Of these the radioimmunoassay and immunohistological assays were investigated and their relative merits will now be discussed.

5.1.8. The Radioimmunoassay.

a) Specificity-The Controls

The introduction of a reliable positive control in this assay was made difficult in the context of this study by the lack of a human antibody which would bind selectively onto breast carcinoma cells. An alternative positive control was used ,utilising the CLA4 cell line and NIBBS serum as described. Whilst not ideal it was hoped that this control would show that the assay worked,that is that it would detect sites where human immunoglobulin was bound onto human cells. In the latter asssay the main problem encountered was that of high background binding. This occurred in all the negative controls described including those in which all but the I^{131} labelled rabbit anti mouse antibody was omitted. It was therefore concluded that this high background was due to nonspecific binding of this antibody to the wells of the plate. Not even complete immersion in blocking buffer overcame this problem. Special attention was paid to ensure that each of the washing stages was performed very thoroughly but this had no effect on abolishing non-specific binding. The high level of background binding remained a problem in this assay and made assessment of the positive controls impossible. The assay was not used routinely, therefore to characterise the reactivity of the McAbs.

b)Sensitivity.

The assay employed in this study was described in detail by Sikora et al.(1983(b)), who stated that it detected immunoglobulin to a level of 20 ug/ml and hence is a sensitive assay.The technique described and used in this assay was made more sensitive by the introduction of more than one antibody.It is possible to conjugate the McAb itself with I^{125} and then layer this onto the antigen(the direct method) but the addition of the second and third antibody stages was shown to increase the sensitivity of the assay by Warenius et al,(1983) who estimated that the addition of a third antibody increased the sensitivity of the technique ten-fold compared with the two stage technique.

c) Relationship to In-Vivo Activity.

One of the main disdvantages of this assay was that it used cultured cell lines of the appropriate type as target antigen. The surface antigens of these cells bear an unknown relationship to those found on tumour cells in actual tumours. This may be especially true for breast cancer cell lines since breast cancer cells are difficult to grow in culture and hence there is a distinct possibility that those cell lines in culture are atypical and may have deletions or expression of new antigens on the cell surface. One alternative to this would be to use freshly prepared suspensions from operative specimens as target cells. It was, however, difficult to produce pure suspensions of tumour cells and the presence of a few macrophages expressing cytophilic immunoglobulin would produce false positive results .

The relationship of the binding pattern of a McAb to cultured cell lines used in the radioimmunoassay to the in-vivo binding pattern of the McAb is unknown. There are therefore several disadvantages to the use of the radioimmunoassay

d) Time and Cost.

The radioimmunoassay as performed in this study was found to be a long and somewhat tedious assay to perform. This could have been improved by the use of multichannel pipettes but the assay remains time consuming.

It was also quite an expensive assay to perform, requiring I^{125} as a tracer and special equipment including a radioactivity room and gamma counter. Whilst the ultimate failure of the radioimmunoassay in this study may be attributed to a problem with the mouse anti human kappa and lambda antibodies there are considerable theoretical and practical considerations which made the radioimmunassay unsuitable for this study.

In spite of these disadvantages the radioimmunoassay has been used quite extensively to determine the activity of McAbs to a variety of tumour types. including melanoma and colon (Steplewski, (1980), lung (Sikora et al.,1981), the ZR75 breast cancer cell line (Hendler et al 1981),breast (Taylor-Papadimitriou et al.,1981) and melanoma (Warenius et al., 1983).It is of interest that while Sikora et al.(1983(a)) found some activity when McAbs were tested against a variety of cell lines by radioimmunoassay this was not confirmed when immunoperoxidase staining of sections of the appropriate tumour were performed. This confirms some of the above

criticisms against using a radioimmunoassay for screening the reactivity of human McAbs to human tumour cells. The assay is of some value when McAbs were orignally produced by immunising mice with cultured tumour cell lines, since one would then anticipate some reaction to these cell lines in the assay. The relationship of this interaction to the in-vivo reactivity of the antibody remains unclear.

5.1.9. Immunohistology.

This type of assay allows sites of McAb/antigen binding to be determined on tissue sections thus allowing the histological sites of this binding to be visualised. Two methods have been described, the immunofluorescence technique and the immunoperoxidase technique. Immunofluorescence is the less useful of the two since it requires special equipment, is difficult to compare sites of fluorescence with the standard histology of the slide,and the fluorescence is not permanent (Taylor, 1978). Whilst techniques for using immunofluorescence to detect McAb/cell binding have been described (Yuan et al.,1982) the technique was not employed in this study because of its theoretical disadvantages.

5.1.10. The Immunoperoxidase Technique.

a) Specificity.

The technique shared with the radioimmunoassay the difficulty of providing a reliable positive control, since this would require a human McAb with specificity for breast cancer cells. The positive control used in this study was a rabbit polyvalent antibody reacting with human epithelial membrane antigen, produced by immunising rabbits with the human milk fat globule membrane

(Sloane et al,, 1981). This antibody was said to bind to the luminal surfaces of normal breast epithelium and to a variety of breast tumours. It was found in this study to provide quite specific staining of both normal and malignant breast epithelial cells and was therefore regarded as an acceptable control.

Negative controls included the use of the HMy2 supernatant and normal human serum in place of hybridoma supernatant, as well as omitting sequentially the various antibodies used in the assay. Once again background binding was a problem and as previously mentioned this background staining was coming from binding of the second stage antibodies to immunoglobulin already present in the tumour tissue section . This background was not seen when the anti EMA control was used since in this case the second stage antibodies were omitted.

Several other causes of background binding in the immunoperoxidase technique have been described. Firstly the presence of endogenous peroxidase within the tissue section was described as a potential problem . Many authors (McGee et al.,1982, Wunderlich et al.,1981, Lansdorp et al.,1980) have advocated treatment of slides with 3%H_2O_2 in methanol to prevent this. Heyderman et al.(1977) stated that 3% H_2O_2 was inadequate to prevent background binding to endogenous peroxidase, and advocated the use of 10% H_2O_2 instead. The value of using 10% H_2O_2 was therefore examined in this study using a series of matched pairs of slides, the one set being treated with 3% H_2O_2 and the other with 10%H_2O_2. No difference in

the degree of background binding between the two groups could be identified and therefore pretreatment of slides with 3%H_2O_2 was continued as standard throughout the course of this study.

A second source of background binding was the nonspecific binding of protein to the tissue sections. It has been recommended that slides are pretreated with an appropriate protein solution to block such binding sites before the assay is performed, Thus Wunderlich et al.(1981) pretreated slides with normal goat serum, Douillard et al,(1980) with foetal calf serum and Naiem (1982) with normal human serum It was important to ensure that the blocking solution would not itself bind to any of the antibodies used in the assay ,for which reason the use of both human and rabbit serum would have been inappropriate in this study. Normal swine serum was used, therefore, as the blocking solution since this should not interact with any of the antibodies used (Taylor 1978).

As has been described the concentrations of all the antibodies used in the assay were adjusted to reduce background binding.

In spite of these precautions it was difficult to reduce the level of background staining, which was attributed to the presence of human immunoglobulin on the tissue sections. This binding occurred both on the fibrous stroma of the breast and on some individual cells which were thought to represent surface immunoglobulin bearing cells .

The difficulties of interpreting immunohistological staining of human McAbs on sections of human tissue because of endogenous

immunoglobulin has been emphasised by Lennox et al. (1982).Interpretation of all slides stained by the immunoperoxidase technique depended therefore upon careful comparison of each slide with the appropriate negative control. For this reason several negative controls were always produced in each batch of slides being stained.

b)Sensitivity.

The sensitivity of the immunoperoxidase technique depends on the method employed (Taylor 1978). The "direct" technique in which the peroxidase enzyme is conjugated directly to the McAb provides a rapid assay system but has the two disadvantages :- a) it is much less sensitive than the indirect technique and b) the conjugation of the peroxidase to the McAb may alter the reactivity of the McAb. (Field 1983).

To increase the sensitivity of the assay the "indirect" technique has been recommended. In this technique the peroxidase is conjugated to a second or subsequent antibody, each additional step increasing the sensitivity of the method several-fold. There remains the problem that conjugating the peroxidase molecule onto one of the antibodies will alter the reactivity of the antibody and for this reason the PAP technique has been recommended (Taylor, 1978, Weij et al.,1983, Field, 1983). This technique uses as its final antibody an antibody raised against the peroxidase molecule , and this antibody is then conjugated with its substrate, peroxidase. This peroxidase-antiperoxidase (PAP) complex can be

bound onto the site of McAb binding using an excess of the bridging antibody (in this case the swine anti-rabbit antibody). The indirect PAP method for performing the immunoperoxidase assay, whilst more lengthy ,offered considerably increased sensitivity and was used as standard throughout this study.

Other factors have been described which affect the sensitivity of the technique. The type of tissue section used may be important. Curran et al (1977) commented that fixation of tissue sections may mask tissue antigens which would otherwise be exposed if frozen sections were used. McGee et al (1982) however performed identical assays on frozen sections and subsequent paraffin sections of the same tissue and found no difference in the binding pattern obtained. This finding was also reported by Hand et al (1983). Posner et al (1982) used viable cells in an attempt to overcome this problem.

In this study a variety of target antigens were used but frozen sections were not used. Paraffin sections of the hybrids "own" tumour and other tumours were used as well as cytocentrifuge preparations of suspensions of cultured breast cancer cell lines and cell suspensions prepared from fresh breast tumour tissues. In general, the definition of stain obtained on the cytocentrifuge preparations was not as good as the paraffin sections.Fixed tissue preparations, or enzyme treated preparations ,do,however, have limitations in the screening of McAbs, since many antigens may fail to survive the fixation process, and enzyme treatment may remove surface antigens and/or expose irrelevant antigens.

The formation of Fc rosettes with tumour cell suspensions was performed to define tumour infiltrating macrophages and to characterise human tumour cells. However the problem of background binding made this technique less successful than was hoped for.

The value of pretreating paraffin sections with trypsin has been emphasised by Curran et al,(1977).Trypsin unmasked tissue antigens which were concealed by the fixation process thus increasing the sensitivity of the technique. This method was employed throughout this study for paraffin sections although no attempt was made to confirm the beneficial effects of trypsin.

The choice of reagent used to mark sites of peroxidase binding may also play a part in determining the sensitivity of the technique. Whilst diaminobenzidine has been a popular choice giving a clear brown stain (Schlom et al.,1980,Naiem et al.,1982) it has the disadvantage of being potentially carcinogenic. Al-Kaissi et al,(1983) described a variety of potential substrates for use in the peroxidase technique. Three-amino 9 ethylcarbizole is not carcinogenic , and was used routinely in this study. Throughout the study this reagent produced clear red staining which was easy to identify against the haemotoxylin counterstain.

c) Relationship to InVivo Activity.

One great advantage of the immunoperoxidase technique is that it can be used to determine the histological site of McAb binding on tumour sections .This can provide valuable information about the distribution of the tumour antigens thus recognised within the tumour and also between various tumours. It can also be used to test sections of tissue which were fixed in the past thus allowing wide comparisons of the activity of the McAb to be made. In addition the record of McAb binding thus produced is permanent.

Using the method described in this study the only disadvantage was the problem of background binding and further investigation is required to overcome this difficulty.

5.1.11. Cytotoxicity Tests.

Cytotoxicity test were not performed in this study. Such tests require that when the McAb binds to the tumour cell it will cause cell death in vitro. Such assays can be complex to develop and often rely on the use of cultured cell lines as targets which is disadvantageous as has been discussed. Such tests are more likely to be of value when investigating the cytotoxic effects of McAb/toxin conjugates.

5.2. RESULTS.

5.2.1. Infection.

At the beginning of this study infection with yeast organisms presented a major problem within the laboratory, in spite of the fact that all glassware and reagents were resterilised and the incubator thoroughly cleaned before the study commenced. Yeast infection was resolved only by the addition of amphotericin B (fungizone) into the culture media. Fungizone is widely used in McAb production (Schlom et al.,1980). A variety of concentrations of fungizone have been used by different workers eg 1ug/ml (Edwards et al.,1982), 2ug/ml (Kearney et al.,1978). In this study fungizone did not appear to inhibit hybrid growth at a concentration of 2.5ug/ml (the manufacturers recommended maximum concentration) and was therefore included in all media throughout the study.

Few studies state that infection has been a problem although Goding (1980) mentioned the problems of mould infection and Epstein et al. (1982) emphasised the problems of bacterial yeast and fungal infection. Whilst the addition of fungizone helped improve the rate of early yeast infection all plates eventually succumbed to some form of infection. The various factors which were thought to contribute to infection of plates in this study fell into two groups, a) those causing bacterial infections and b)those causing infection by yeasts and moulds..

a) Bacterial Infections.

Bacterial infections tended to occur early (mean 8.12 days after fusion) and to affect two or more plates from the same fusion experiment at the same time- eg FE 50/1-4 all developed

bacterial infection on day 7. Bacterial infection was always observed the day after fusion or the day after the cells had been fed, and did not appear to spread to adjacent plates within the CO2 incubator. It did however appear in most if not all of the wells of the affected plate.

From these observations it was concluded that bacterial infection was caused by direct contamination of the plate during fusion or feeding since all plates of each fusion would be fused or fed together at the same time. The precise source of bacterial contamination was never identified . Careful attention was paid to sterile technique whilst fusing and feeding plates. In addition a continuous suction device was devised for use in removing one ml of spent medium when the plates were being fed and these measures reduced the rate of bacterial contamination. This is demonstrated by the fact that no bacterial infection occurred after FE 51.

b) Yeast and Mould Infection.

The pattern of yeast and mould infection differed from bacterial infection. Yeast and mould infections occurred relatively late (mean 52.78 and 49.39 days respectively after fusion), and bore little relationship to feeding the plates The infection tended to affect only one or two wells in the plate in the first instance, and these were usually at the periphery of the plate. Infection also spread to adjacent plates in the incubator, rather than to plates of the same fusion. For these reasons it was concluded that yeast and mould infection occurred because of airborne contamination within the incubator (although yeast contamination may also have occurred in some cases when the plate was being

fed). The CO_2 incubator relied for its humidity on a tray of water placed in the bottom of the incubator . The warm moist conditions thus produced were an ideal breeding ground for these organisms which could then spread in droplet form. One infected well would then act as a source of infection for adjacent wells and plates. Attempts to arrest established infection and save the plate by clearing out the infected well and rinsing it several times with 70% alcohol were unsuccessful.

Attempts to prevent infection were only partially successful.Initially the incubator was emptied and cleaned weekly but this caused more disturbance of the plates and an increase in the rate of infection . Regular cleaning of the incubator was therefore abandoned at an early stage.The plates were stacked in chronological order in the incubator and the close stacking of the plates may have facilitated cross infection (plate 4). It might have been useful in retrospect to store the plates of each fusion in separate plastic boxes. The addition of an antiseptic to the water in the incubator was avoided since this might have inhibited hybrid growth.

The incidence of infection may also have been related to the layout of the laboratory. Ideally all work should be carried out in a clean tissue culture laboratory, with the CO_2 incubator and inverse phase microscope beside the hood so that there would be minimal disruption of the plates. Whilst all fusion experiments and cell culturing work were performed in a class II microbiological safety cabinet this hood was situated in a room which had a permanent dust problem from the roof (plate 5).The CO_2 incubator was situated in a separate room in the midst of a

variety of other equipment and so plates would be exposed to contamination every time the door of the incubator was opened. In addition plates had to be carried along an outside corridor between hood,incubator and microscope, again increasing the risk of contamination (plates 6). Thus the physical layout of the laboratory was somewhat unsuitable for the work of this study but these factors could not be altered easily.

PLATE 4

The inside of the CO_2 incubator, to demonstrate the close stacking of culture plates.

PLATE 5.

The Envair Class II microbiological safety cabinet in which all sterile cell fusion and culture work was performed.

PLATE 6.

The CO_2 Incubator (Leec), surrounded by other equipment.

5.2.2. Overall Results.

In this study 25 hybridomas were obtained from 231 successful plates (5544 wells). This gave a hybrid rate of 0.108 hybrids per 24 well plate, or 0.004 hybrids per well. Alternatively the rate can be expressed as 0.008hybrids per 10^6lymphocytes used. These various expressions of hybrid rate were all necessary to allow comparison with other studies some of which are now summarised for comparison.

Kohler et al (1976) estimated that approximately one hybrid was obtained for every 10^6 lymphocytes used for fusion using a mouse/mouse system. Schlom et al (1980) obtained 301 hybrids from 1460 wells,a rate of 0.206 hybrids per well. However of these only 52 secreted immunoglobulin (0.035 hybrids per well) and only 23 remained stable immunoglobulin secretors(0.015 hybrids per well). These results were obtained in a human lymphocyte/mouse myeloma cell fusion. Further experience of this system increased the number of hybrids to 505 from 2060 wells (0.245 hybrids per well) although only 62 secreted immunoglobulin (0.03 hybrids per well)and only 23 were stable in culture (0.011 hybrids per well). (Wunderlich et al 1981). Yuan et al (1982) using a mouse/mouse

fusion system reported hybrid growth in 80% of the wells seeded. Papsidero et al.(1983) obtained approximately 3000 hybrids from a mouse/mouse fusion system although only about 5% of these secreted immunoglobulin of any interest.

The reported fusion efficiency of the HMy2 cell line in different hands varies and is sometimes difficult to discern!

Edwards et al,(1982) described a hybrid efficiency of one hybrid per 10^6to10^8 lymphocytes used. Sikora et al.(1982(b)) reported 71 hybrids from 5 patients from the fusion of glioma infiltrating lymphocytes with the HMy2 cell line. The same author (Sikora et al.1983(a)) obtained 55 hybrids from 180 patients by the fusion of tumour node lymphocytes with the HMy2 cell line. Olsson et al. (1984) obtained 305 immunoglobulin secreting hybrids from 1436 wells of a human human fusion,fusing PWM stimulated peripheral blood lymphocytes with the RHL4 cell line, but only one hybrid secreting immunoglobulin of required specificity survived in culture. Warenius et al.(1983) described hybrids appearing in 43-55% of wells using HMy2 in which lymphocytes were prestimulated with pokeweed mitogen compared with only 12-19% of wells when pokeweed mitogen was not used. However it was then revealed that only 12 hybrids grew adequately in prolonged in-vitro culture.

Experience with other human myeloma cell lines has been reported as follows.

Croce et al.(1980(a)) obtained hybrids in 20 out of 24 wells using the GM1500 cell line and peripheral blood lymphocytes.Shoenfield et al,(1982) obtained 108 hybrids from 4254 wells (0.02 hybrids per well), using the GM4677 subline of the GM1500 cell line, and either peripheral blood or spleen

lymphocytes. Kozbor et al.,(1982) obtained one hybrid per 10^5 lymphocytes when these had been transformed with Epstein Barr virus compared with one hybrid per 10^7 lymphocytes with no EBV transformation,using a variant of the GM 1500 cell line. Olsson et al.(1983) described 39% of 53 fusions producing hybrids with the RHL4 cell line and 19% of fusions using the SKO 007 cell line. Overall results however produced only 21 antibody secreting hybrids from 100 fusions . Glassy et al.(1983) obtained a range of 0.54 to 2.9 hybrids per 10^6 lymphocytes depending on the type of lymphocytes used, using the UC729-6 myeloma line. Overall it appears that mouse/mouse fusions were the most efficient hybrid producers, and that mouse human fusions were more efficient than human human fusions at producing hybrids although many of these resulting hybrids are unstable in culture.

The hybrid rate from human/human fusions is lower than that of mouse /human or mouse/mouse fusions and it varies considerably. Experience with the HMy2 cell line suggests that it has a rate of hybrid production comparable with other human myeloma lines (Cote et al.,1983). The results obtained in this study compare rather poorly with other published results confirming the observation by Sikora et al.,(1982(c)) that human hybrid production varied in efficiency from laboratory to laboratory. The results obtained in this study compare with the lower end of the hybrid rate reported by Edwards et al.,(1982). Some of the factors which may be responsible for this low rate of hybrid production will be discussed in the following paragraphs.

5.2.3. Pokeweed Mitogen (PWM).

The use of PWM to prestimulate lymphocytes prior to fusion is becoming more widespread and the majority of studies suggest that this step increases the rate of hybrid production. Shoenfield et al.(1982) described the stimulation of peripheral blood lymphoctes with a 1/100 dilution of PWM for 48 hours as giving a much increased rate of hybrid production. Olsson et al, (1983) stimulated peripheral blood lymphocytes with pokeweed mitogen at 2.5 ug/ml for 5-7 days and this significantly improved the rate of hybrid production. Warenius et al. (1982) stimulated node lymphocytes with PWM at 20ug/ml for 5 days ,resulting in improved rates of hybrid production. Sikora et al. (1983(a)) stated ,however, that PWM did not increase the rate of hybrid production although no data was given to support this. Olsson et al,(1983(a)) stimulated peripheral blood lymphocytes at 2.5 ug/ml/10^6 cells for 5 days. It is believed that prestimulation of lymphocytes with PWM is a useful step towards increased hybrid production. The various regimes used for stimulating lymphocytes suggests that further investigation is required to decide upon the optimum stimulation regime and this itself may vary depending on the source of lymphocytes used.

From this study tritiated thymidine uptake gave reproducible results when measuring the effects of PWM stimulation and was the preferred method. Neither of the two regimes used to stimulate lymphocytes prior to fusion were the optimum regimes

suggested by the data from the tritiated thymidine uptake experiments. The two regimes selected for use in this study were chosen on the basis of other mitogen experiments performed previously in the laboratory (data not presented) . The two regimes selected were sufficiently different to allow reasonable comparison between them.The tritiated thymidine uptake experiments were performed concurrently with the fusion experiments as node lymphocytes became available. A criticism of this study is that the tritiated thymidine uptake experiments should have been performed first before carrying out the fusion experiments , so that the optimum regime for lymphocyte stimulation could be selected. This may indicate one cause of the poor hybrid rate obtained in this study. As has been discussed the level of H^3 uptake at five days was uniformly lower than at two days in spite of increasing the feeding regimes. This suggests that many cells which were dividing at two days were no longer doing so at five days . This was confirmed by the cell counts which showed a lower number of cells present at five days than at two. The plateau effect of the dose-response curve using various concentrations of PWM appeared to occur at the 8ug/ml level. This is thought to represent the point at which all lymphocytes capable of being stimulated by PWM have indeed been stimulated. From this data it appears that a regime of stimulating lymphocytes at 16ug/ml for two days might be the most beneficial. This regime remains to be tested in fusion experiments, and to this extent the study of the effects of PWM remains incomplete. In addition in the experience of this study and previous work done in the

laboratory , numerous axillary lymph nodes were found to be hypo-reactive and sometimes totally anergic following PWM stimulation. This may have contributed to the lack of success using PWM.

5.2.4. Feeder Cells.

The use of feeder cells to improve hybrid efficiency appears to have arisen by the chance observation that the wells of a mouse mouse fusion which produced most hybrids were those in which active macrophages could be seen (Fazekas et al (1980(a)), these cells presumably having come from the spleen cell suspension. Feeder cells have been used widely and successfully to improve the rate of hybrid production, although the mechanism by which this occurs remains unclear.

Fazekas (1980(b)) suggested that feeder cells acted first by phagocytosing the large amount of possibly toxic cell debris from the large number of dying cells in each well. Thereafter they acted as feeder cells producing and secreting substances beneficial to hybrid growth. Reading (1982) suggested that the use of feeder cells reduced the need for medium changes and that this decreased the risk of contamination. Lernhardt et al ,(1978) pointed out that feeder cells can be equally effective if derived from different species .

Two main types of feeder cells are described as increasing the success rate of mouse-mouse fusions, these being mouse peritoneal macrophages and foetal mouse thymocytes although other cell types have also been investigated.

Mouse peritoneal macrophages were found to increase hybrid

efficiency in mouse-mouse fusions by a number of authors (Shulman et al.,1978, Fazekas et al.,1980 (a)) and also in mouse-human fusions (Edwards et al.,1980,Lernhardt et al.,1978). Thymocytes were likewise found to be of value in mouse-mouse fusions and also in the cloning of murine hybrids (Schlom et al 1980).

Astaldi et al,(1980,1981) compared the efficiency of these cells with the supernatant of a culture of human endothelial cells derived from the umbilical cord. In this study using mouse-mouse fusions , the human endothelial cell supernatant (HECS) was found to be very efficient at increasing hybrid efficiency. The active ingredient in HECS was shown to be of molecular weight greater than 50,000, stable in storage and not a mitogen to peripheral blood lymphocytes. The active ingredient was thought to bind to hybridomas and acted equally well on mouse-mouse and mouse-human hybridomas. This would suggest that part of the activity of feeder cells is the secretion of growth promoting substances .Less is known about the effects of feeder cells on human-human hybrids.

Edwards et al (1982) reported that mouse peritoneal macrophage feeder cells increased the hybrid rate fivefold, but Sikora et al. (1982(a)) stated that feeder cells did not improve hybrid production,both studies using HMy2 as parent myeloma. Kennett et al,(1978) used irradiated human foetal fibroblasts to increase the hybrid frequency of mouse-human hybrids. Olsson et al,(1983) reported the benefits of using human monocytes and thymocytes in human-human fusions using the SK0 007 and RHL4 cell lines although the data presented is scarce. Brodin et al.(1983) described the use of peripheral blood monocytes as feeders to

improve the cloning efficiency of human hybridomas.

In this thesis it was decided to investigate the addition of human feeder cells to hybrid cultures. It was decided that it would be inadvisable to use feeder cells of another species since this study was investigating the production of human human hybrids and the addition of cells from another species may allow overgrowth of cells of that species.

In choosing feeder cells it was decided to use close equivalents of those cells which had been found to be beneficial in murine work . Thus independently of Olsson et al. (1983) peripheral blood monocytes were chosen since they were readily available, were known to have phagocytic . ability and resembled the use of murine macrophages.Thymocytes were likewise chosen because of the documented benefit of murine thymocytes and a constant supply of these was available (courtesy of Mr K.Reid, Cardiac Surgeon,Royal Hospital for Sick Children, Edinburgh). The failure of feeder cells to improve the rate of hybrid production was disappointing and suggests that further investigation is required to define the precise qualities of feeder cells which are important to hybrid growth, and to investigate more fully those human feeder cells which are of use. Since the failure of feeder cells in this study to improve hybrid production may be related to other problems with the fusion system it is suggested that peripheral blood monocytes and thymocytes from young children are worthy of further investigation.

5.2.5. Cell Numbers.

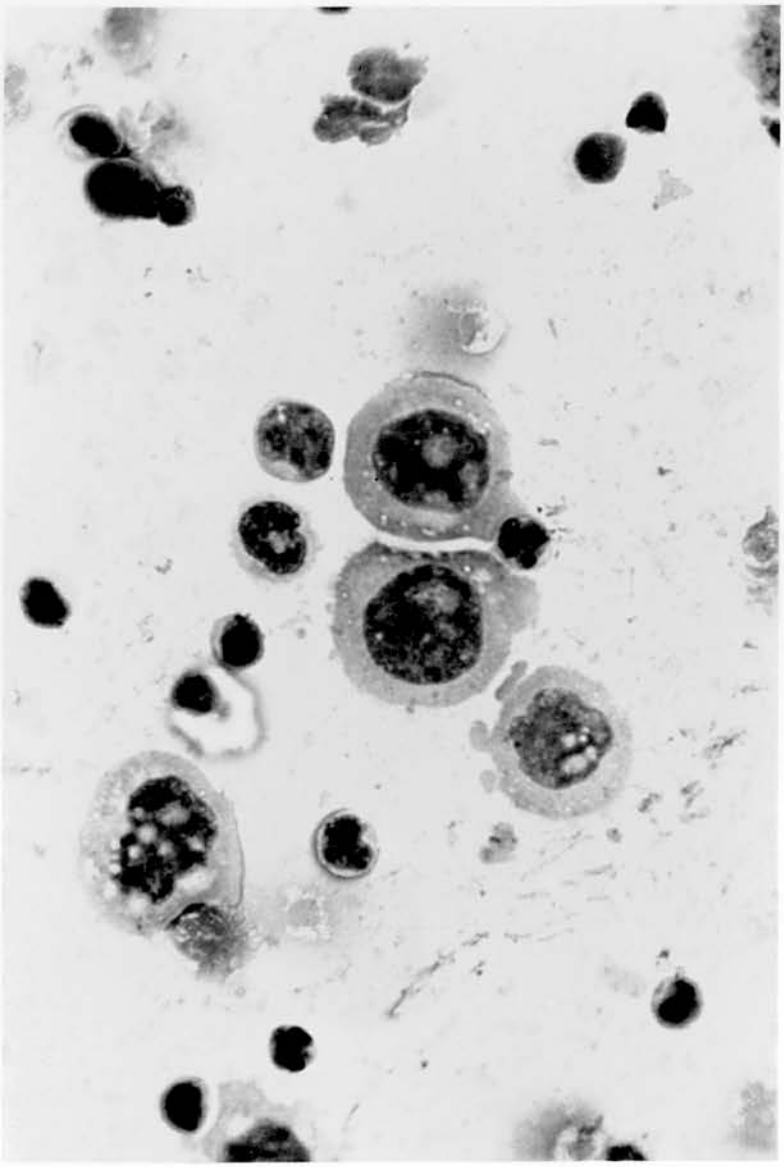
The ratio of myeloma and lymphocyte cells in fusion experiments varies widely in different studies. Indeed it has been suggested that the initial excess of spleen cells used by Kohler and Milstein came about more by chance than design. Other studies have used a large variety of lymphocyte/myeloma ratio. Sikora et al. (1982(b)) used a 3:1 excess of myeloma cells in the production of human human hybridomas although this was said to be due to a relative lack of available lymphocytes. Kozbor et al.(1982), Cote et al (1983), Gigliotti et al.(1984) used equal numbers of lymphocytes and myeloma cells .Glassy et al.(1983)3 used a 2:1 ratio of lymphocyte to myeloma , Wunderlich et al,(1981) a 4:1 ratio. Edwards et al.(1982), Warenius et al,(1983) both used a 10:1 lymphocyte/myeloma cell ratio.

Relatively little attention has been paid to investigating the benefits of these various ratios, and so it was decided to investigate the effects of a 1:1, 5:1 and 10:1 lymphocyte:myeloma ratio on hybridoma production. The following factors may be of importance in determining the optimum cell ratio.When the two cell types are mixed in suspension in the PEG solution they will freely move freely and collide with each other. Successful fusion requires that one lymphocyte and one myeloma cell collide and fuse together. Assuming the cells are of equal size one would expect that a 1:1 ratio would optimise the possibility of successful collisions.However the HMy2 cell is much larger than the lymphocyte (plate7). Hence an excess of lymphocytes may increase the chances of successful fusion.

PLATE

Cell suspension of a mixture of HMy2 cells (large cells) and axillary node lymphocytes (small cells) to demonstrate their respective sizes.

(Giemsa stain, magnification x100).



In addition successful fusion requires the coming together of two actively dividing cells. The proportion of lymphocytes actively dividing at the time of fusion is much less than the HMy2 cell line and therefore an excess of lymphocytes seem to be required for successful fusion.

Whilst no advantage was found in this study by altering cell ratios, there seems to be sound theoretical grounds for using an excess of lymphocytes and for these reasons a 10:1 lymphocyte:HMy2 ratio was used for all subsequent fusions in this study.

5.2.6. Selective Medium.

It has been suggested that azaserine may be more appropriate than aminopterin for use in selective medium (Edwards et al., 1982) In this series azaserine was shown to be more efficient than aminopterin when the overall results were compared. Unfortunately this did not occur when direct comparison between the two was made. The benficial effect of azaserine was therefore inconclusive since other factors may have influenced the successful fusions using azaserine. Whilst accepting this criticism, the data does suggest that azaserine is more useful in terms of hybrid production and this requires further controlled investigation. It may be that the number of plates involved in the controlled series was too small to demonstrate the difference. The use of azaserine has been used routinely by other authors (Edwards et al.,1982, Larrick et al.,1983).

5.2.7. Hybrids.

a) Time of Appearance.

The time after fusion at which hybrids appeared varied widely (range 24-69 days) but there was no observable difference in the growth pattern of hybrids obtained either early or late after fusion. Therefore all plates were maintained in culture for as long as possible until infection supervened.

The variation in time at which hybrids appear may explain in part the low hybrid rate of this study. Plates were included in the results of this study if they survived more than 24 days in culture (this being the earliest date at which a hybrid appeared). Thus a plate which developed,say a yeast infection at day 30 would be scored as negative whereas had the plate survived it might have produced a hybrid at a later date. Thus a plate which would have become a positive was scored as a negative,-ie the scoring system employed in this study allows a potentially high number of false negative results. The alternative would be to employ a higher threshold at which plates were included in the results,which would have the effect of reducing the number of possible false negatives but of decreasing the number of plates in the study. This effect is demonstrated on table 5 (page 110) in

which the effects of adopting different times of scoring plates on the hybridoma rate are shown. This manner of data presentation can be misleading in one respect ,in that plates may produce hybrids and then become contaminated before they reached this threshold, allowing a falsely high positive error to be introduced. As an example if plates were to be scored only if they survived forty days or more in culture and a plate produced 2 hybrids on day 30 and developed yeast contamination on day 35 the hybrids would be counted but the plate regarded a a technical failure.

Perhaps a more appropriate method of presentation would be to raise the minimum time in culture to 40 days but to include in these results any plate which produces a hybrid before this time. The effect of doing this has been demonstrated in table 5. In conclusion the criteria by which plates are included in the results will determine to some extent the hybrid rate. Whilst the method adopted in this study allows the maximum number of plates to be included ,it will produce the highest number of false negative plates and may artificially reduce the overall hybrid rate. When compared with other methods of scoring plates however ,it appears to offer the fairest way of collating results. As table 5 shows altering the results in this way brings the overall hybrid rate in this study closer to those of other studies already quoted.

b) Culture Conditions.

The effects of varying the various parameters of the fusion experiment on the rate of hybrid production have already been discussed. Of particular interest is the relationship between hybridoma production and the date on which the corresponding

fusion experiment was performed. From table 20 there appears to be definite clusters of dates of fusions which were most efficient at producing hybrids, which is of interest since fusion exeriments were carried out regularly every week throughout the course of this study. This would suggest that there may be other factors operating at these particular times which produce successful fusion. Such factors may include the quality of the FCS (although all batches were tested as described), or the quality or the pH of the particular batch of PEG in use at that time. It has not been possible to identify in retrospect any particular factor which may have been responsible for this phenomenon, but it does demonstrate how little is known of the precise factors necessary for cell fusion.

c) Hybrid Growth.

Once established,hybrids grew rapidly in culture and could be frozen and thawed reliably. It was disappointing that so many hybridomas failed to survive transfer from the 24 well plates to the 25ml tissue culture flasks. Strenuous efforts were made with the later hybrids to dilute them very gently. Most of the hybridomas were probably lost at this stage because of the difficulties of maintaining the pH of a small amount of medium in these flasks in spite of gassing with CO_2, and this was improved by changing the medium from TCM-B to TCM-A at this stage.

5.2.8. Immunoglobulin Secretion.

From the agglutination assay all hybrids were found to secrete IgG which was to be expected since this is secreted by the HMy2 line. A few hybrids also secreted IgM giving evidence of new immunoglobulin secretion by these hybrids at least. As has been

discussed, the agglutination reaction did not provide a means of accurately defining the immunoglobulin secreted.

In addition the supernatants were only tested for IgG and IgM in the first instance, on the assumption that they would secrete one of these classes of immunoglobulin.

Cote et al (1983) detected IgA secreted by hybridomas of axillary node lymphocytes fused with HMy2 cells. Following this report attempts were made to identify IgA in the hybridoma supernatants, but none was found.

The level of immunoglobulin secretion by the hybridomas in this study was of the order of 0.01-0.82 ug/ml of supernatant. Edwards et al.,(1982) reported an immunoglobulin secretion of 0.5-0.8ug/ml and Sikora et al.,(1983(a)) a rate of 1-5ug/ml.The parent ARH-77 line secreted immunoglobulin of the order of 12.1 ug/ml of Ig per 10^6 cells.Other human hybridomas are reported as secreting approximately the same amount of immunoglobulin. Olsson et al.(1980) obtained 3-11 ug/ml from hybrids of the U266 line. Kozbor et al.,(1983) obtained 3-6ug/ml from hybrids of the GM1500 line. This was described as being 13 to 18 times greater than the concentration of immunoglobulin secreted by EBV transformed lymphocytes on their own. Glassy et al.(1983) obtained 3-9 ug/ml from hybrids of the U729-6 line. Human-mouse hybrids are described as secreting a slightly higher immunoglobulin level (10-26ug/ml by Schlom et al.,1980, 8.3ug/ml by Nowinski et al 1980). The agglutination assay was found to be a valuable means of screening hybridoma supernatant and measuring the concentration of immunoglobulin present. This study confirmed the low level of immunoglobulin secretion by human-human hybridomas and this remains a problem.

The finding that some hybridomas secreted both IgG and IgM on the basis of the agglutination assay was of interest. Assuming that each parent cell was committed to the production of only one antibody this provided evidence that the hybridoma was secreting antibody derived from both parent cells. It is not possible on the basis of this assay to ascertain whether the hybridomas were secreting two different classes (IgG and IgM) of immunoglobulin simultaneously or whether the agglutination assay was detecting immunoglobulin chains from both parent cells which had reformed to produce a new antibody. It is planned to further investigate these supernatants at a later time to determine whether, in fact, the hybridomas were secreting two different antibodies simultaneously.

5.2.9. Cloning.

Hybridomas obtained in this study were found to clone readily by limiting dilution without the addition of feeder cells. at a concentration of one to two cells per well. In addition hybrids which secreted both IgG and IgM were found to do this through several cloning procedures indicating that hybridomas from the HMy2 cell line clone satisfactorily.

Whilst the importance of regular cloning of hybridomas to maintain levels of immunoglobulin secretion is emphasised in many studies, no hybridoma was kept in continuous culture for longer than 3 months since none of them secreted McAbs of sufficient interest to merit prolonged investigation. Thus once a hybridoma was grown in culture and sufficient supernatant gathered for characterisation the hybridoma was frozen and stored.

5.2.10. The Reactvity of Hybridoma Supernatants.

As has been discussed already in this chapter, the radioimmunoassay was found to be an unsatisfactory method of assessing the reactivity of McAbs and was abandoned. The immunoperoxidase method was developed therefore as the technique of choice for testing the reactivity of McAbs to breast cancer cells.

Whilst the staining obtained using the immunoperoxidase technique on cytocentrifuge preparations of tumour cells was not very clearly defined there was no discernible selective binding of McAbs to these cells.

The interpretation of staining on tumour sections was difficult since this required careful comparison of each slide with the corresponding negative control slides. This background staining presented in three ways. Firstly moderate staining was seen in much of the fibrous connective tissue of the breast , this presumably being due either to the presence of free immunoglobulin or possibly a non-specific reaction to the antigenic epitopes on the connective tissue. This staining tended to be spread uniformly throughout the connective tissue. Secondly isolated cells within tumour cell collections were seen to stain very darkly around the cell membrane. This was presumed to indicate the presence of surface immunoglobulin bearing cells possibly macrophages or plasma cells within the tumour. These cells are difficult to distinguish histologically from tumour cells. Thirdly staining was occasionally seen in some areas at the luminal edge of normal epithelium. The reason for this part of the background stain is unclear. These three forms of background staining often made

the interpretation of McAb staining difficult. This was particularly true of the cells within tumour cell aggregates which stained darkly. However no McAb staining pattern resembled the positive anti EMA stain and after careful and repeated examination of the slides, no McAb was seen to bind selectively to the breast cancer cells.The pattern of distribution of immunoglobulin found within breast tumours was demonstrated by Richman (1976) using immunofluorescence to demonstrate the binding of anti-human immunoglobulin antibodies to sections of both benign and malignant breast tissue. The pattern of immunoglobulin binding in that study was similiar to that demonstrated in this present study.

SECTION 6.

CONCLUSIONS.

CONCLUSIONS.

The production of human McAbs with reactivity against human tumour cells raised expectations that these antibodies would provide new avenues for treating cancer. Whilst initial studies concentrated on the production of murine McAbs, these may be unsuitable for clinical use because of their relative lack of specificity and because of possible hypersensitivity reactions with repeated usage. This study has explored the production of autologous human monoclonal antibodies with reactivity against human breast carcinoma cells. It has demonstrated both the feasibility of producing such McAbs and the problems associated with their production.

There appear to be two main problems which are currently limiting the clinical application of monoclonal antibodies, these being 1) the technical difficulties associated with cell fusion and human monoclonal antibody production, and 2) the lack of precise knowledge of the nature and distribution of tumour antigens within tumour cell populations. This study aimed to investigate the technical problems of monoclonal antibody production, and also reviewed the available evidence about tumour antigens.

The difficulties of producing human monoclonal antibodies because of the low rate of hybridoma production and the low rate of immunoglobulin secretion has been well documented. The low rate of hybridoma production by cell fusion was investigated in this study, by studying various factors involved in

the fusion experiment. The factors studied were a) the prestimulation of lymphocytes with pokeweed mitogen ,b) the addition of feeder cells into the fusion experiment, c) the use of azaserine in place of aminopterin in the selective medium, and d) the alteration of the cell numbers used for the fusion experiment. Prestimulation of lymphocytes with PWM did not , in this study, improve the rate of hybridoma production. This was disappointing since other studies had demonstrated benefit from PWM. The failure of PWM in this study may be due to the regimes used, or the overall hybridoma rate was to low to allow any discernible benefit to be demonstrated. In future it is planned to use EBV transformation of lymphocytes prior to fusion to stimulate selective growth of the B-cell population. The addition of feeder cells also produced no improvement in the hybridoma rate in this system. Very little is known about the mechanisms by which feeder cells have produced their beneficial effect in murine McAb production and feeder cells require further detailed investigation. The use of azaserine in place of aminopterin did produce an overall improvement in the rate of hybridoma production in this study and is recommended for routine use. A 10:1 lymphocyte/myeloma ratio was used routinely since no benefit was obtained from altering this ratio. Of particular interest was the finding that hybridomas arose in clusters from fusion experiments performed within short periods of time. The factors essential for successful fusion are reviewed in the text and it is concluded that there is little information available about the mechanisms

involved in cell fusion. There is a need for more basic research to improve our understanding of cell fusion so that the rate of human hybridoma production may be improved. The rate of immunoglobulin secretion by hybridomas is a function primarily of the myeloma cell parent and was not further investigated in this study.

Problems were encountered with both the radioimmunoassay and the immunoperoxidase method used in this study to determine sites of binding of the McAb to breast carcinoma cells. Whilst the immunoperoxidase method was the method of choice in this study, there remains a need for the more accurate detection of sites of McAb binding to breast carcinoma cells which closely resembles the in-vivo activity of the McAb.

The nature and distribution of tumour antigens within breast carcinoma cell populations remains unclear. It has been documented in the literature that there is a heterogeneity of antigenic expression as detected by McAbs within breast carcinoma cell populations and that there is some antigenic cross- reactivity of antigens between tissues from benign and malignant breast tumours. It is concluded that the ultimate clinical value of human anti-breast carcinaoma antibodies will be determined by the nature and distribution of these antigens. For the foreseeable future it appears that the main role of anti-breast cancer McAbs will be in the definition of these antigens.

APPENDICES.

APPENDIX 1.

MATERIALS AND REAGENTS USED.

1. Tissue Culture Media.

   a) Tisssue Culture Medium (TCM) A

      R.P.M.I. 1640 (Ros well Park Memorial Institute)

R.P.M.I.1640	10.39g
$NaHCO_3$	0.70g
Penicillin	100,000 units.
Streptomycin	0.10g.

      Distilled Water to Make 1000ml.

   b) TCM-B

      Dulbeccos Modified Eagles Medium (DMEM).

DMEM	13.65g
Na HCO_3	3.50g
Penicillin	100,000 units
Streptomycin	0.10g
Fungizone	0.25mg

      Non Essential Amino Acids:-10 ml of x100 solution.

      Distilled water to make 1000ml.

2. Foetal Calf Serum (FCS) (Gibco)

   Heat inactivated at 56°C for one hour.

3. Polyethylene Glycol (PEG)

   Sigma cat. no p3515. Molecular Weight 1000.

PEG Solution 1.

PEG 2.5g autoclaved at 15 psi for 20 minutes,

Solution cooled to 60°C

0.75ml of Dimethyl Sulfoxide (DMSO) and 2.75 ml of TCM-B added.

5% Na HCO_3 used to adjust pH to 7.8.

PEG Solution 2.

PEG 2.5g autoclaved at 15 psi for 20 minutes

Solution cooled to 60°C.

7.5ml of TCM-B added

5% $NaHCO_3$ used to adjust pH to 7.8.

(DMSO - Sigma cat. no D5879).

4. Pokeweed Mitogen Sigma cat. no.L9379.

5. Selective Media.

HAT x100 solution.

Hypoxanthine	650mg.
Aminopterin	10mg.
Thymidine	195mg.

0.01N NaOH to make 500 ml.

HAzT x100 solution.

Hypoxanthine	140mg
Azaserine	4mg
Thymidine	50mg

0.01N NaOH to make 100ml.

6. Radioimmunoassay.

Poly-L-Lysine Sigma cat.no.P1399.

Mouse anti huma kappa and lambda chain antibodies.

Bethseda Research Lab cat.no. 31030 & 31040.

Rabbit anti mouse antibody DAKO, Denmark Z109.

7. Ficoll Hypaque.

Ficoll 400	63.5g
Hypaque Sodium	100.0g

Distilled water to make 1000ml. (Density 1.0770)

8. Phosphate buffered Saline.

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.1g
KH_2PO_4	0.2g

Distilled water to make 1000ml

9. Immunoperoxidase Method.

Tris Buffered Saline.

Tris Buffer	6.05g
1N HCl	40ml

Distilled water to make 1000ml.

Acetate Buffer

1M Sodium Acetate	50ml
1N HCl	10ml

Distilled water to make 200ml.

Aminoethyl Carbizole

AEC	40mg
N,N Dimethyl Formamide	10ml

Antibodies (all DAKO,Denmark)

Rabbit anti Human IgG	cat no A290
Rabbit anti Himan IgM	cat no A091
Swine anti Rabbit	cat no Z196

Rabbit peroxidase/antiperoxidase cat. no.Z113.

Swine serum obtained from local abbatoir and heat inactivated at 56°C for one hour.

10. Miscellaneous

Collagenese Ia Sigma cat. no C9891

Trypsin Difco.

Trypsin Inhibitor Sigma cat. no T9003.

11. Cultured Cell Lines.

LICR/LON/HMy2

Myeloma cell line used for cell fusions.

Reference :- Edwards et al 1982.

MCF-7

Breast carcinoma cell line derived from pleural effusion of patient with breast carcinoma.

Reference :- Soule et al 1973.

MDA-MB 231

Breast Carcinoma cell line obtained from pleural effusion of patient with metastatic breast cancer.

Reference :- Cailleau et al 1974.

CLA_4

Human lymphoblastoid cell line

Reference :- Steel 1972.

DET 6.

HeLa subline derived form sternal bone marrow of lung cancer patient.

Reference :- Espmark 1975.

APPENDIX 2.

Summary of patients from whom axillary node lymphocytes were obtained for use in this study.
Number of Patients = 70.

Mean Age 51.75 years (Range 33-81).
Axillary Operation Performed (in Conjunction with Surgery to the Breast Tumour).
Axillary Sampling (Lowest 5 Axillary Lymph Nodes Removed).....13.
Lower Axillary Dissection (Clearance of Axillary Nodes up to the level of the Axillary Vein).....20.
Axillary Clearance (Removal of All Axillary Lymph Nodes).....37.
Patients in Whom Subsequent Histological Examination Demonstrated The Presence of Axillary Node Metastases in One or More Nodes.....23.
Patients with No Axillary Metastases.....47.
Screening Procedures.
All patients had the following screening procedures performed pre-operatively:-
Chest X-Ray
Bone Scan
Liver Scan
Haematological and Biochemical Profile.
In all these patients the above investigations were all found to be normal.

APPENDIX 3.

FIGURE 5

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 271

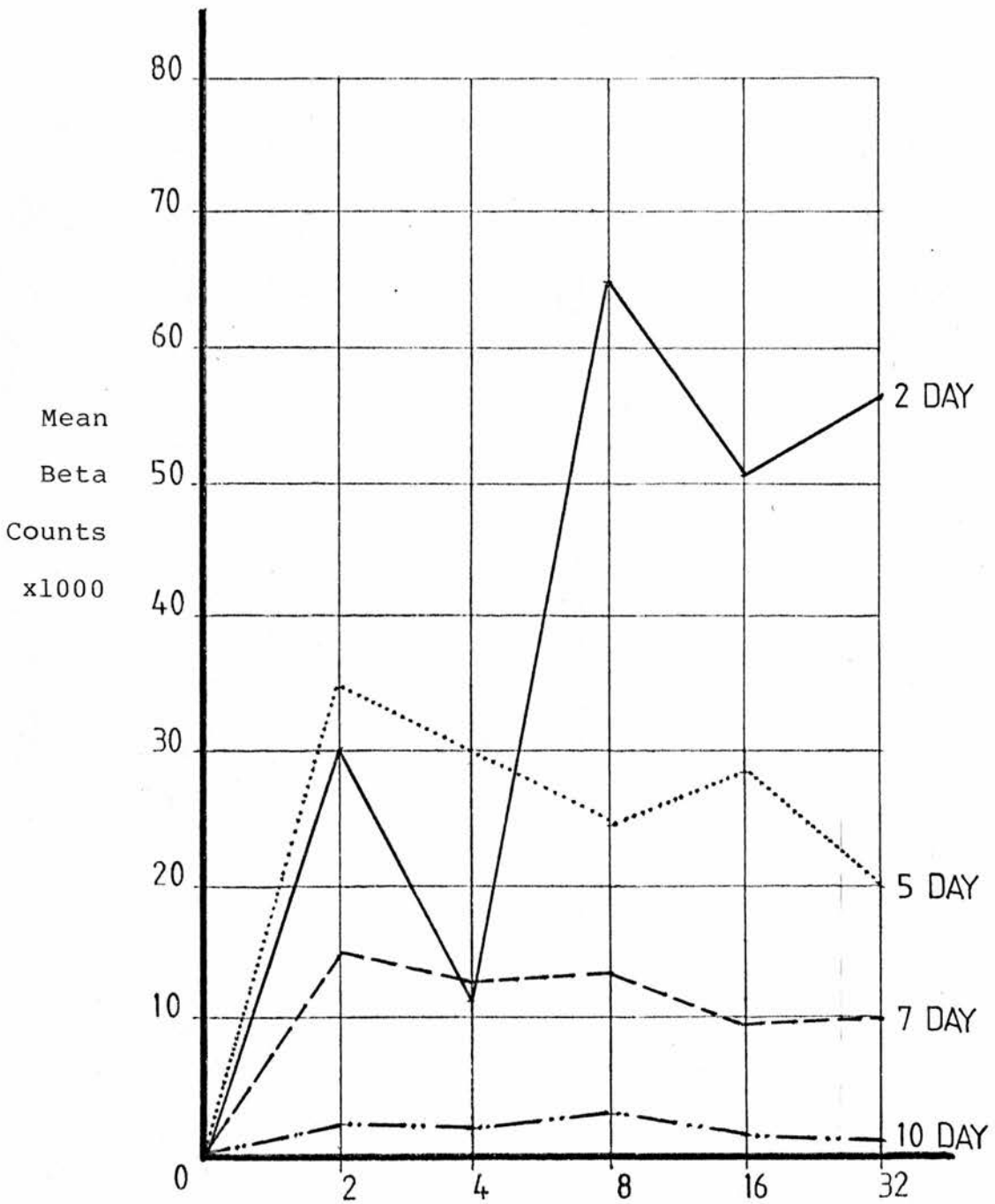


FIGURE 6

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 272

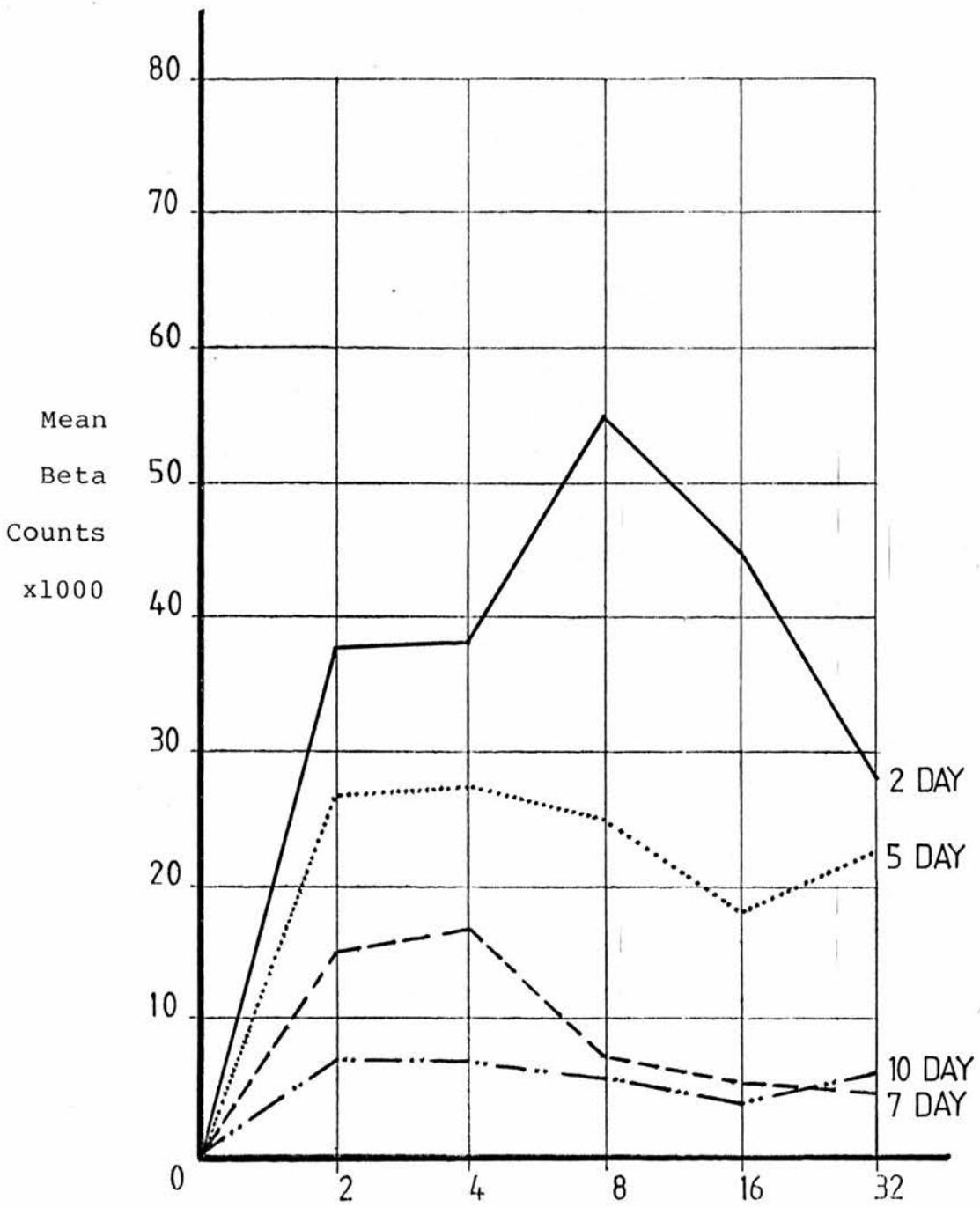


FIGURE 7

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 273

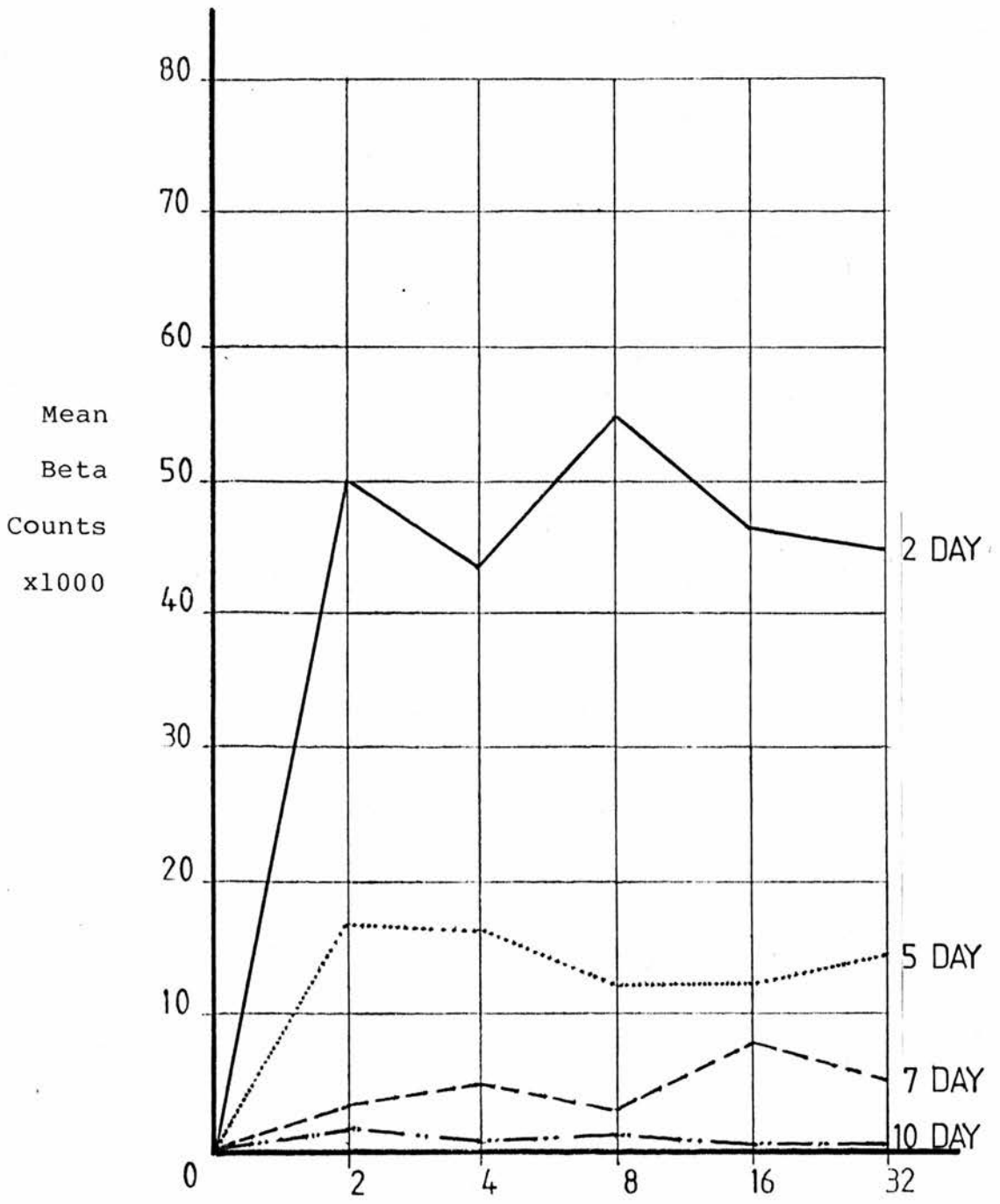


FIGURE 8

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 274

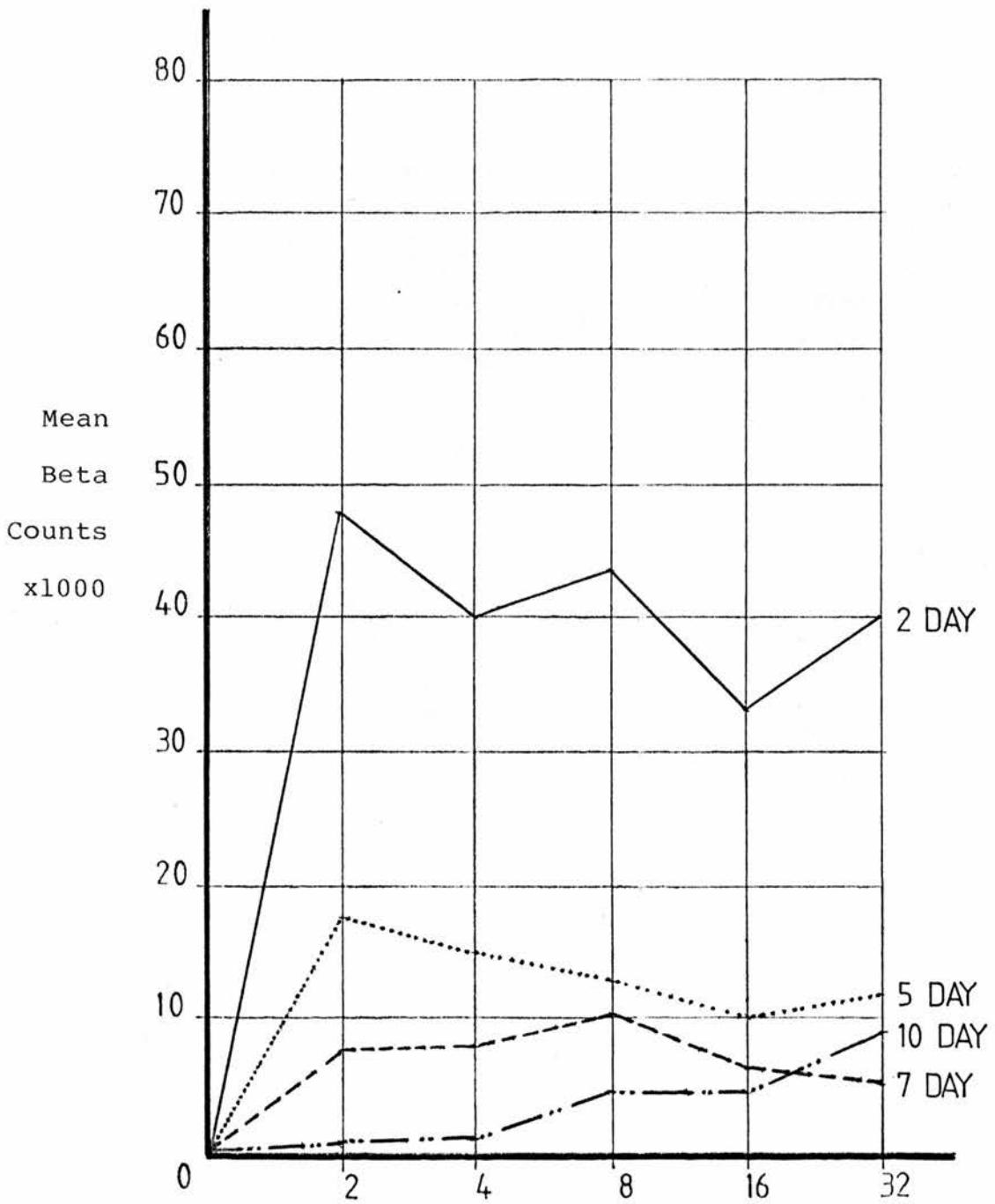


FIGURE 9

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 275.

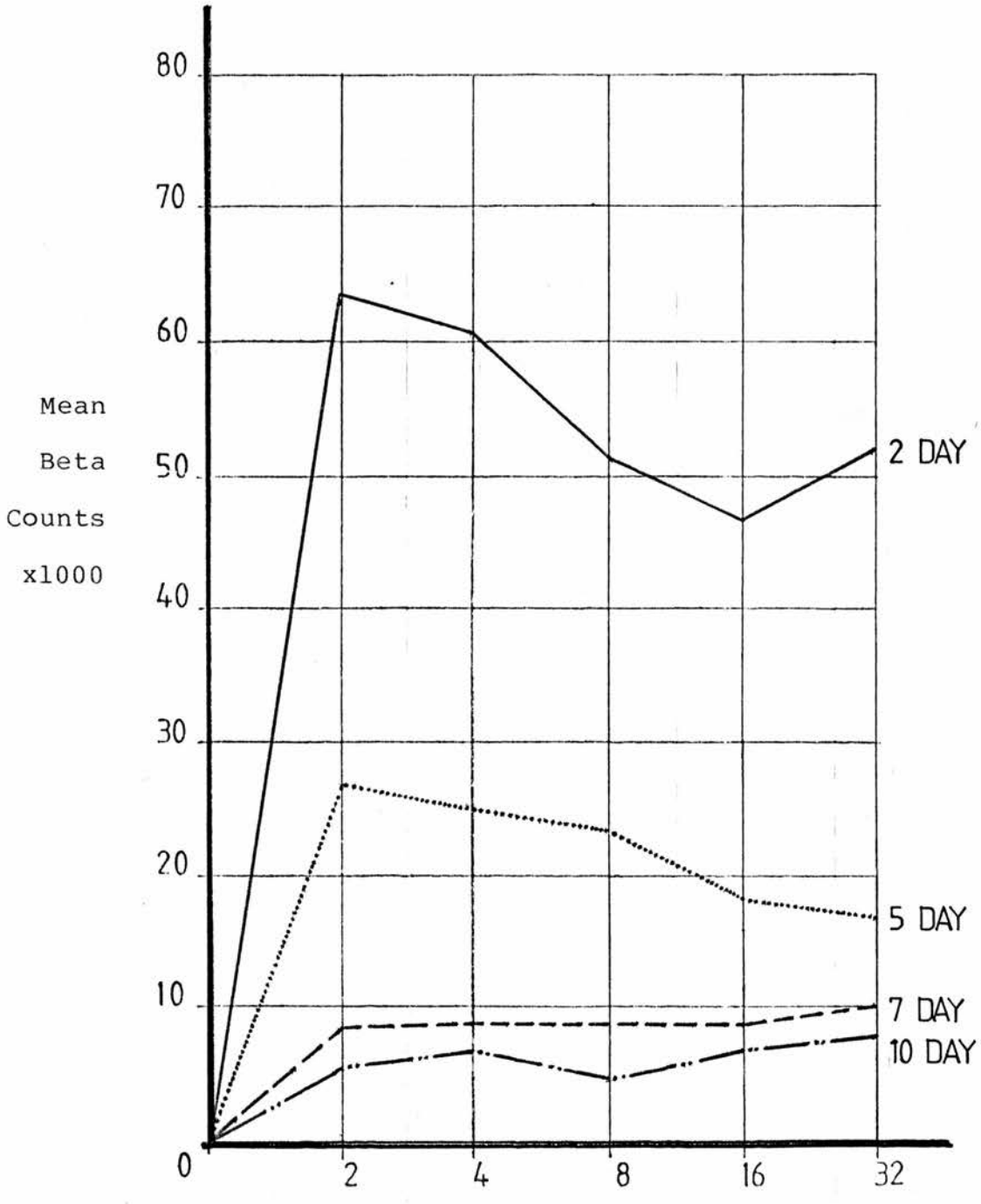


FIGURE 10

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 282.

10% FCS + PWM Feeds.

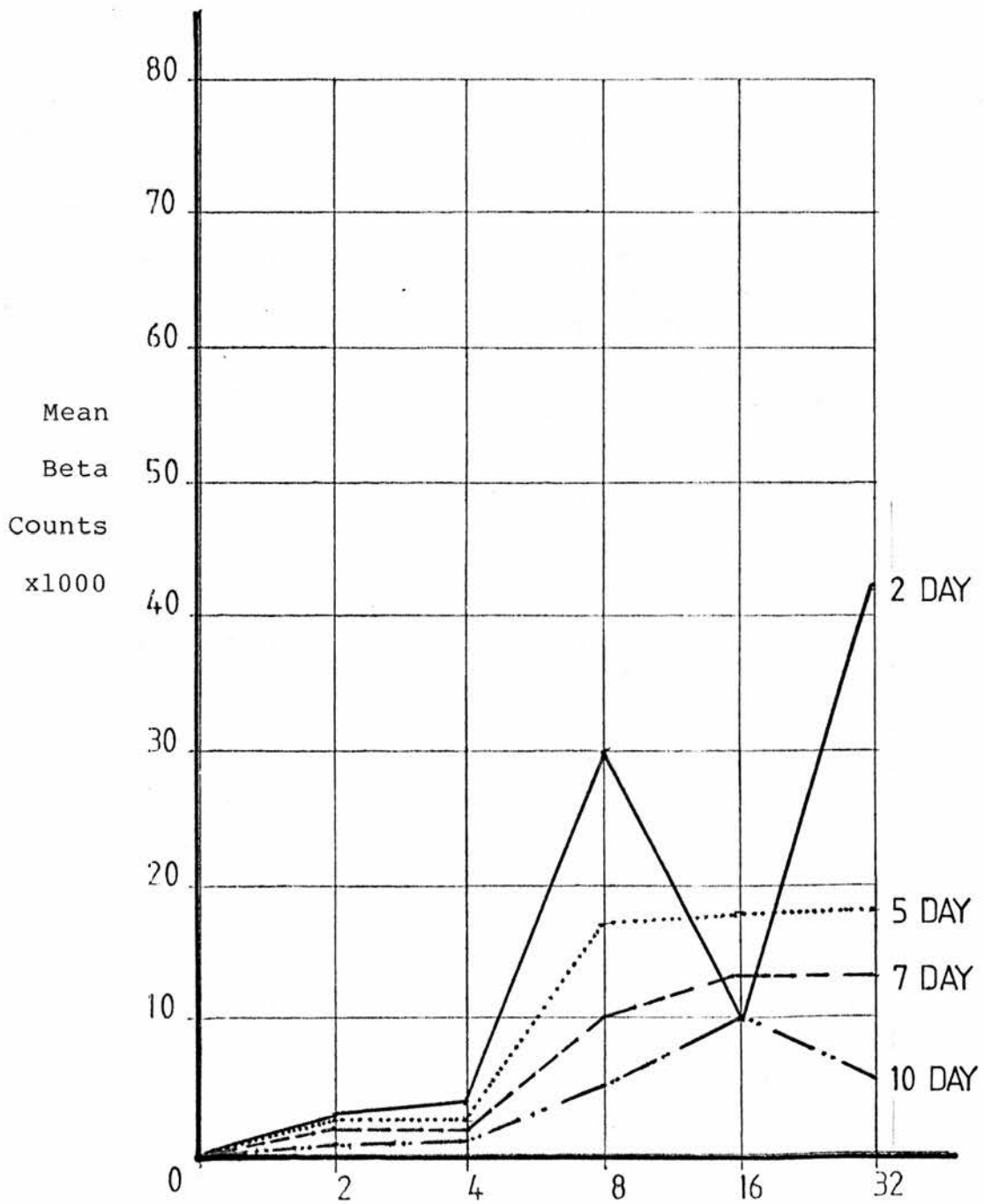


FIGURE 11

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 282.

10% FCS Feeds.

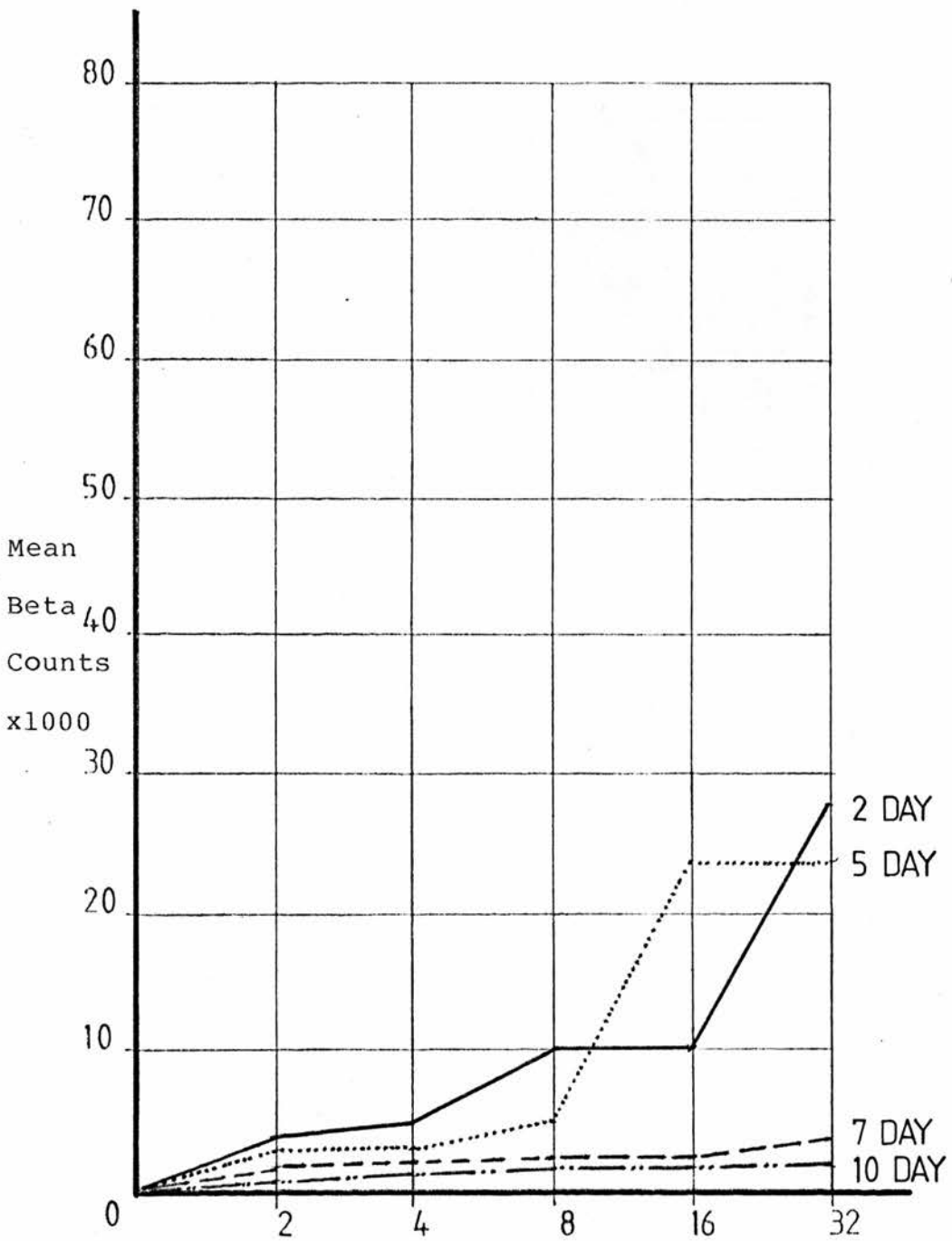


FIGURE 12

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 286

10% FCS + PWM Feeds.

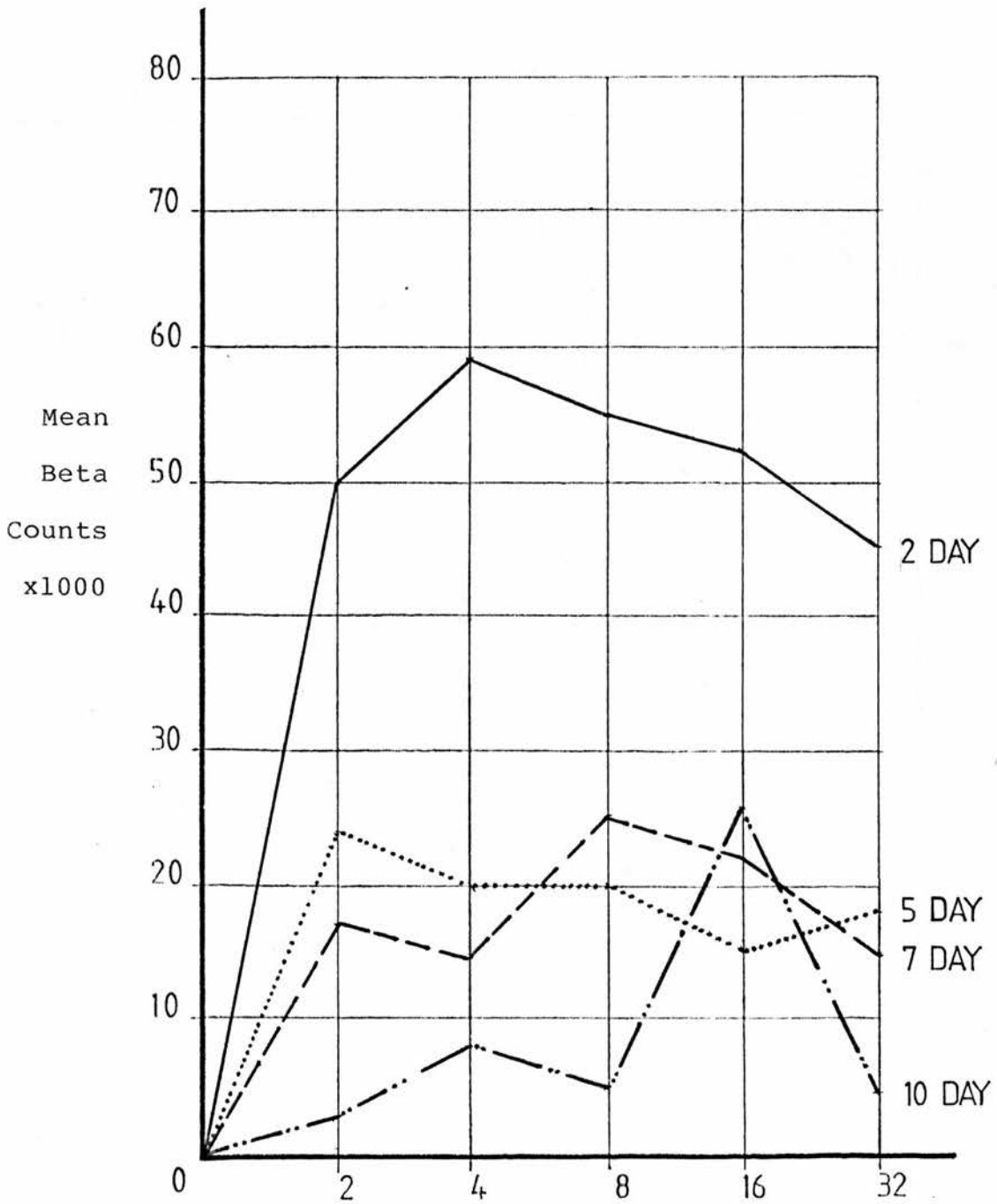


FIGURE 13

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 286.

10% FCS Feeds.

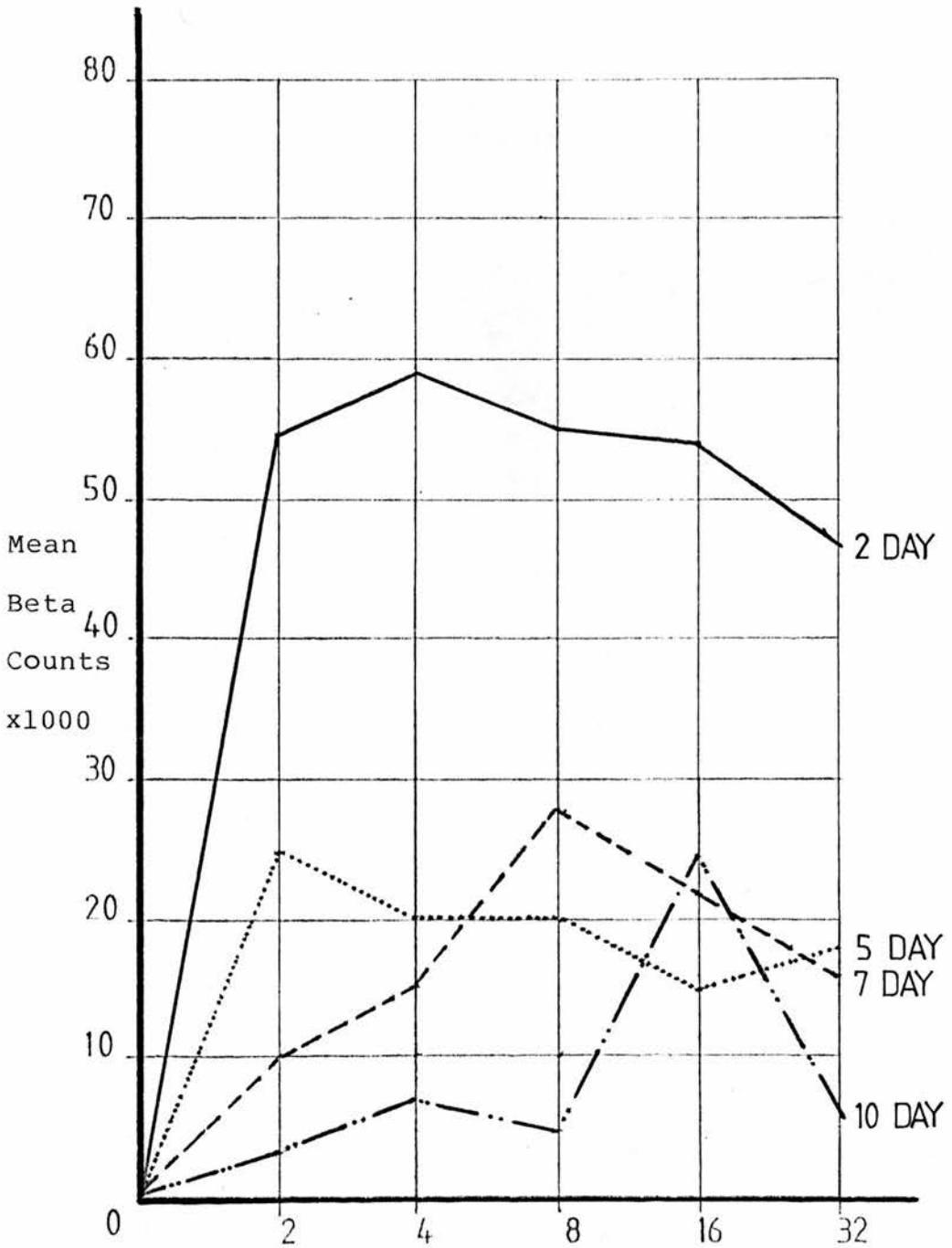


FIGURE 14

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 288

10% FCS + PWM Feeds.

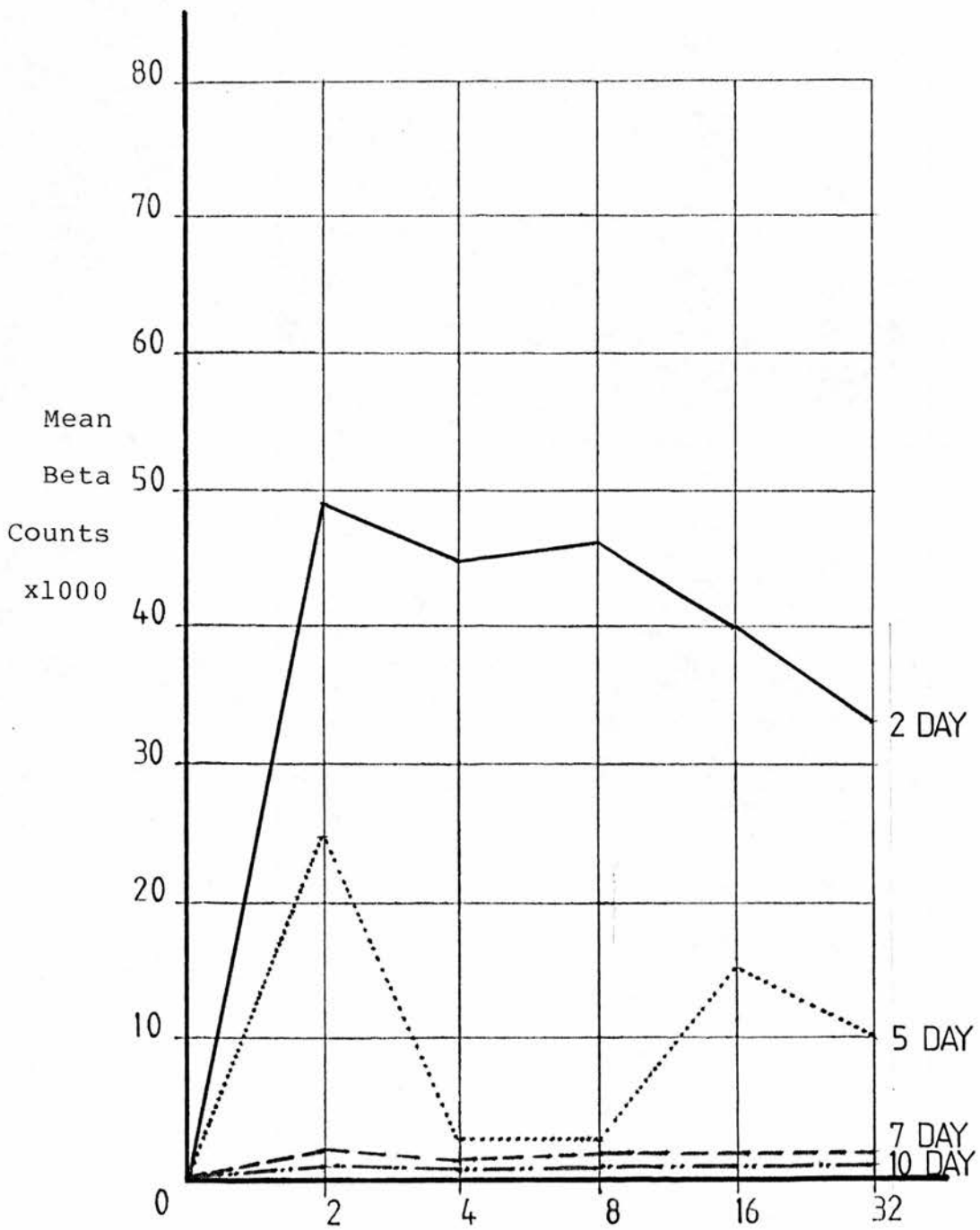


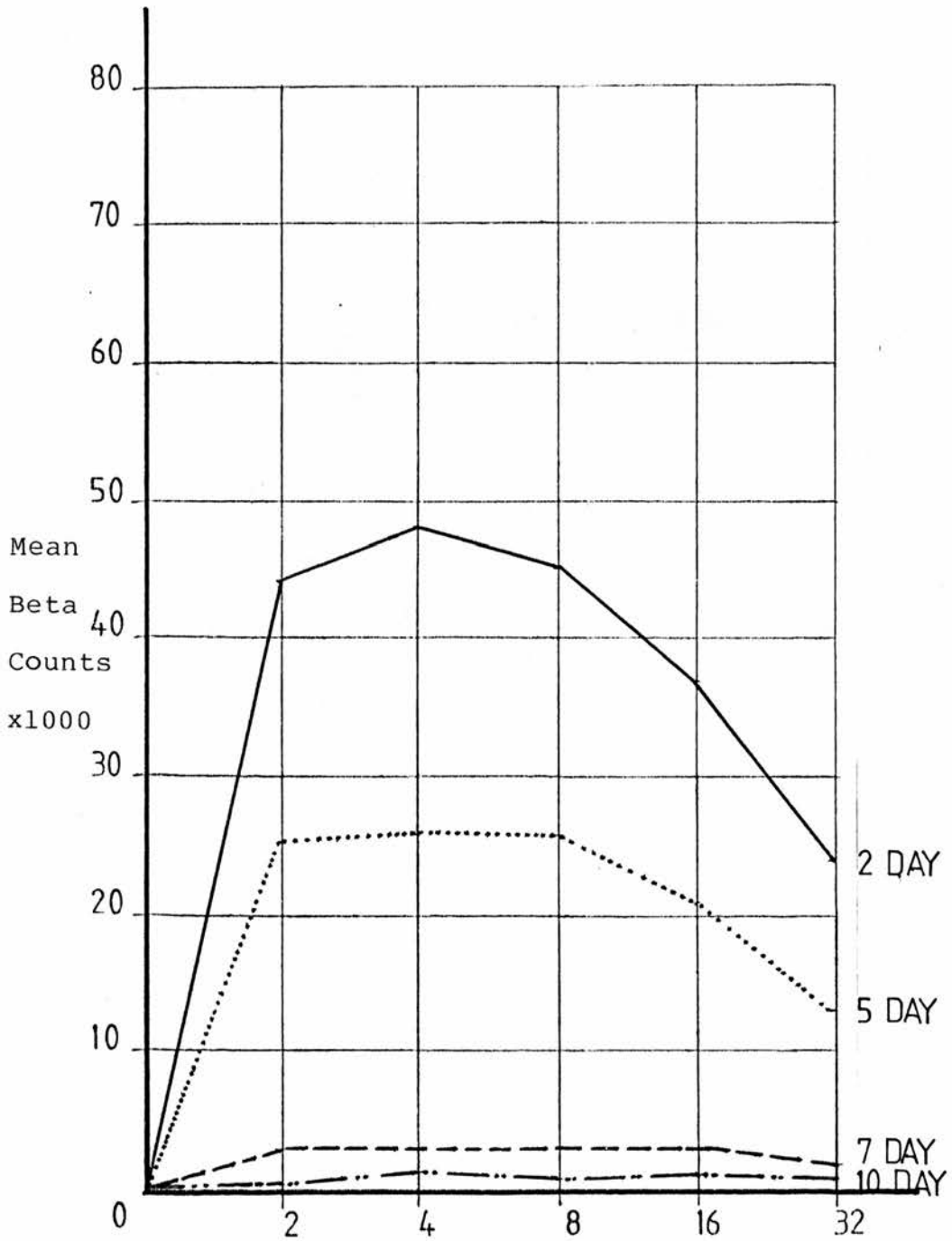
FIGURE 15.

Pokeweed Mitogen Stimulation of Lymphocytes.

Preliminary Experiments.

Tritiated Thymidine Uptake Experiment using T.N.L.E. 288

10% FCS Feeds.



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